“Tis better to have loved and lost. Than never to have loved at all.”

Alfred Lord Tennyson.

University of Alberta

Functional dissection of the role of *scalloped* in *Drosophila* wing and eye development

by

Leola Chow ©

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Abstract

The focus of this thesis is to further our understanding of an evolutionarily conserved transcription factor, Scalloped (Sd), and how it can interact with a nuclear protein called Vestigial (Vg) to form a selector complex to organize *Drosophila* wing fate. The possible role of Sd in *Drosophila* eye morphogenesis was also examined.

With respect to wing development, previous studies had discovered that Sd provides the DNA binding component while Vg serves as the activating component of the Sd-Vg complex. Vg requires Sd to enter the nucleus and the relative concentration of the two proteins is vital to the proper development of the wing. Sd may be divided into the N-terminal domain, TEA DNA binding domain, Vg interacting domain (VID), linker domain (intervening region between the TEA DNA binding domain and the VID), and the C-terminal domain. These domains were determined by sequence alignment with the human homolog of Sd, Transcription Enhancer Factor 1 (TEF-1) or via *in vitro* experimentation. To understand the mechanism of wing gene activation by the Sd-Vg complex, and to examine the defined domains of Sd in an *in vivo* assay, various Sd constructs with deletions or interruptions in these domains were tested for their ability to rescue two recessive viable wing alleles of *sd* (sd^{ETX4} and sd^{58d}). The results are consistent with the idea that the TEA DNA binding domain binds DNA, while the VID and the C-terminal domain are required to form a stable complex with Vg. The linker region was found to serve an unknown yet vital role in wing development, while the N-terminal domain is not necessary for the development of this tissue.

The collection of Sd constructs was also subjected to an over-expression assay in the *Drosophila* eye. It was predicted that similar to the situation in the wing, Sd possibly

interacts with an unknown factor for proper eye morphogenesis. Again, the linker domain of Sd serves an important yet unknown function in eye development.

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List of abbreviations

A/P	anterior-posterior
CBP	cAMP-responsive element binding (CREB)-binding protein
D/V	dorsal-ventral
GTF	general transcription factor
MF	morphogenetic furrow
NLS	nuclear localization signal
R	photoreceptor
RS	restriction site
SCRA	Sveinsson's Chorioretinal Atrophy
TBP	TATA box binding protein
TEA domain	DNA binding motif of Sd
TF	transcription factor
VID	Vg interacting domain

List of genetic symbols

ap	*apterous*
ci	*cubitus interruptus*
dac	*dachshund*
Dl	*Delta*
Dll	*distalless*
Dlw	*dorsal wing*
dpp	*decapentaplegic*
en	*engrailed*
ey	*eyeless*
eya	*eye absent*
eyg	*eye gone*
fng	*fringe*
hh	*hedgehog*
hth	*homothorax*
iro-c	*iroquois gene complex*
N	*Notch*
nina	*neither inactivation nor after potential A*
ptc	*patched*
sd	*scalloped*
Ser	*Serrate*
so	*sine oculis*
Su(H)	*Suppressor of Hairless*
TEF-1	*Human Transcription Enhancer Factor-1*
toy	*twin of eyeless*
tsh	*teashirt*
vg	*vestigial*
vgBE	*vg boundary enhancer*
Vgl-1	*Human Vg like-1*
vgQE	*vg quadrant enhancer*
vn	*vein*

w	*white*
wg	*wingless*
y	*yellow*

Chapter 1.1: General Introduction

One of the most intriguing questions in the field of developmental biology is how a fertilized egg can give rise to an entire organism. In particular, how are cells organized to become specific structures? *Drosophila melanogaster* serves as an excellent model organism to study this question for several reasons. Flies are easy to maintain and culture in the laboratory. They have a short life cycle; it takes approximately 10 days for a fertilized egg to develop into a fly at 25^{O}C (Demerec, 1950; Demerec, 1967). In addition, the entire *Drosophila* genome has been sequenced which makes sequence alignment of genes and sequence motifs possible. Many *Drosophila* genes such as *decapentaplegic* (*dpp*), a transforming growth factor β homologue, *hedgehog* (*hh*), a secreted signaling protein, and *wingless* (*wg*), a member of the Wnt family of morphogens are implicated in various developmental pathways and diseases in higher organisms (reviewed in Ingham and McMahon, 2001; McMahon, 1992; Nusse and Varmus, 1992; Whitman, 1998) and much of the general information learned in *Drosophila* can be extrapolated to these organisms.

The development of the *Drosophila* embryo involves segmentation of the embryo along the anterior-posterior (A/P) axis. This involves a cascade of gene expression that divides the embryo into three thoracic and eight abdominal segments (reviewed in Ingham and Martinez-Arias 1992; St Johnston and Nusslein-Volhard, 1992). The *Drosophila* embryo also establishes a dorsal-ventral (D/V) axis involving a network of gene expression (reviewed in Morisato and Anderson 1995; Steward and Govind 1993). In addition, there are the field-specific selector genes that regulate the formation and/or patterning of an entire structure such as the *Drosophila* wing (Garcia-Bellido, 1975). For example, the *eyeless* (*ey*) gene is required for eye formation (Halder et al., 1995), and the *scalloped* (*sd*) and *vestigial* (*vg*) products are required for wing fate (Halder et al., 1998; Simmonds et al., 1998). The focus of this thesis will be on an evolutionarily conserved transcription factor known as Sd (Campbell et al., 1992), and how it can interact with Vg to form a field-specific complex to organize wing fate. Whether Sd also has a role in eye development was also studied.

A basic summary of both *Drosophila* wing and eye development and how these adult structures are developed from the embryo and the developmental pathways involved will be presented. In addition, how transcription factors can regulate transcription in eukaryotic cells is also discussed.

1.2 Development of *Drosophila* adult structures

Drosophila adult structures derive from imaginal discs which are epithelial cell layers that consist of undifferentiated, proliferating cells (Auerbach, 1936). Imaginal discs originate as clusters of cells in the embryonic ectoderm (Garcia-Bellido and Merriam, 1969; Wieschaus and Gehring, 1976). They grow inside the body cavity until the larva becomes a pupa, at which point they evaginate and fuse and ultimately form the body wall and appendages (Fristrom and Fristrom, 1993). Nine pairs of imaginal discs form the head, thorax, and thoracic appendages, and a medial disc forms the genitalia (Anderson 1972; Cohen et al., 1993). The abdominal epidermis comes from separate cell clusters called "histoblast nests" (Roseland and Schneiderman, 1979), while distinct imaginal rings give rise to the respective salivary glands, gut, and trachea (Hartenstein, 1993). The positions of these various imaginal and precursor cell collections are shown in Figure 1.1. Limb primordia are believed to be established shortly after the blastoderm stage (Wieschaus and Gehring, 1976; Cohen et al., 1990), by which time the embryo has already been "sub-divided" for specific fates via the segmentation genes (reviewed in Akam, 1987, Ingham, 1988). These primordia are established by two signals. *wg* is expressed as a stripe anterior to the parasegment boundary in the embryonic ectoderm (Baker, 1987; Cohen et al., 1993) and *dpp* is expressed in a lateral stripe running perpendicular to the cells expressing *wg* (St. Johnston and Gelbart, 1987; Cohen et al., 1993). The cells in the vicinity of the intersection between Wg and Dpp become specified as limb primordia (Figure 1.2) (Cohen et al., 1993). A *Drosophila* wing primordium can first be visualized as a discrete cluster of *vg* expressing cells in the second thoracic segment of the stage 12 embryo (Carroll et al., 1995; Hartenstein, 1993; Williams et al., 1991). The wing imaginal disc is comprised of about 20 cells when it is formed during embryonic development (Lawrence and Morata, 1977). These cells proliferate during the third larval stage to generate a disc of about 50000 cells in the late third instar (about 96 hours after hatching) (Bryant and Levinson, 1985) which evaginates to become the adult wing. A fate map of the wing is provided in Figure 1.3 (Bryant, 1975).

Specification of the eye-antennal primordium also requires *wg*, since the eye-antennal disc cannot be recovered from *wg* mutants (Simcox et al., 1989). The pair of eye-antennal discs is situated at the posterior end of the dorsal pouch in the newly hatched larva (Chen, 1929; Madhavan and Schneiderman, 1977) and consists of about 20 cells per disc in the embryonic blastoderm (Garcia-Bellido and Merriam, 1969). By the

Figure 1.1: The location of the imaginal discs in a mature third instar larva. The wing imaginal disc is underlined in red and lies within the second to third thoracic segments. The eye-antennal disc is also underlined in red and is situated in the first to second thoracic segments. In this diagram, both female and male reproductive structures and only one copy of each pair of imaginal discs are shown for simplicity. Dorsal structures are on the left and ventral structures are on the right. Diagram modified from Bryant and Levinson, 1985.

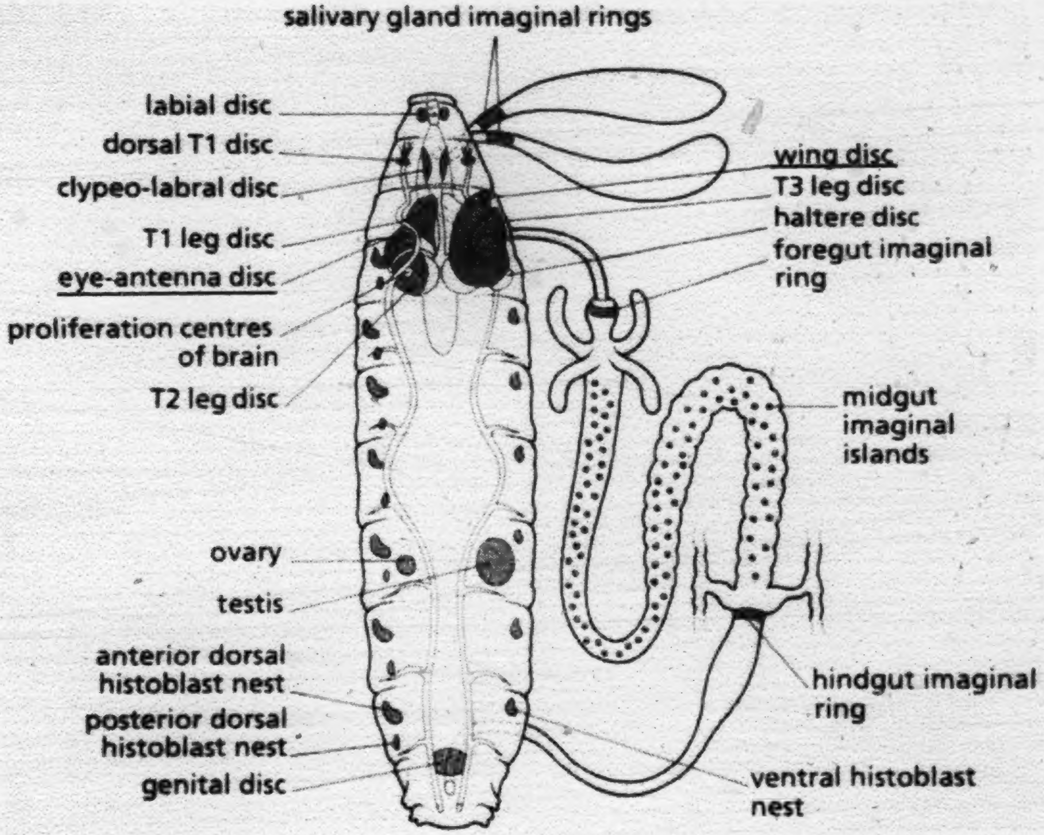
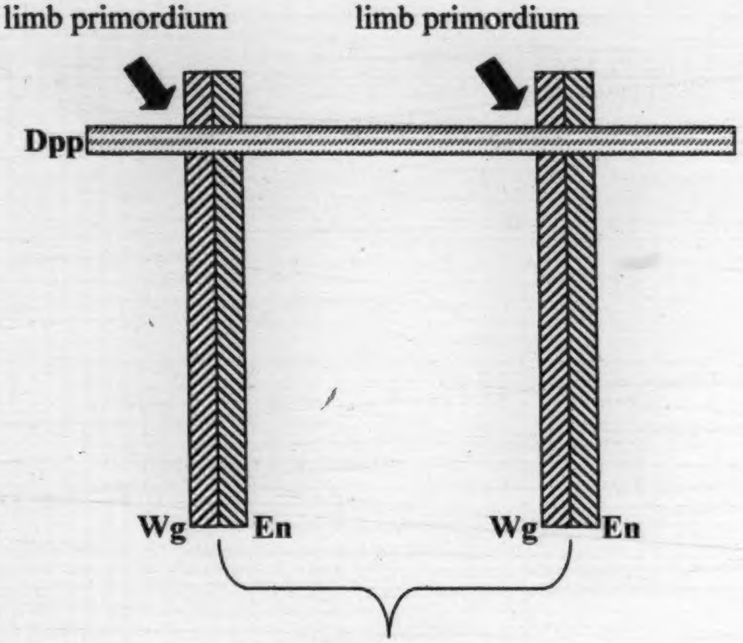
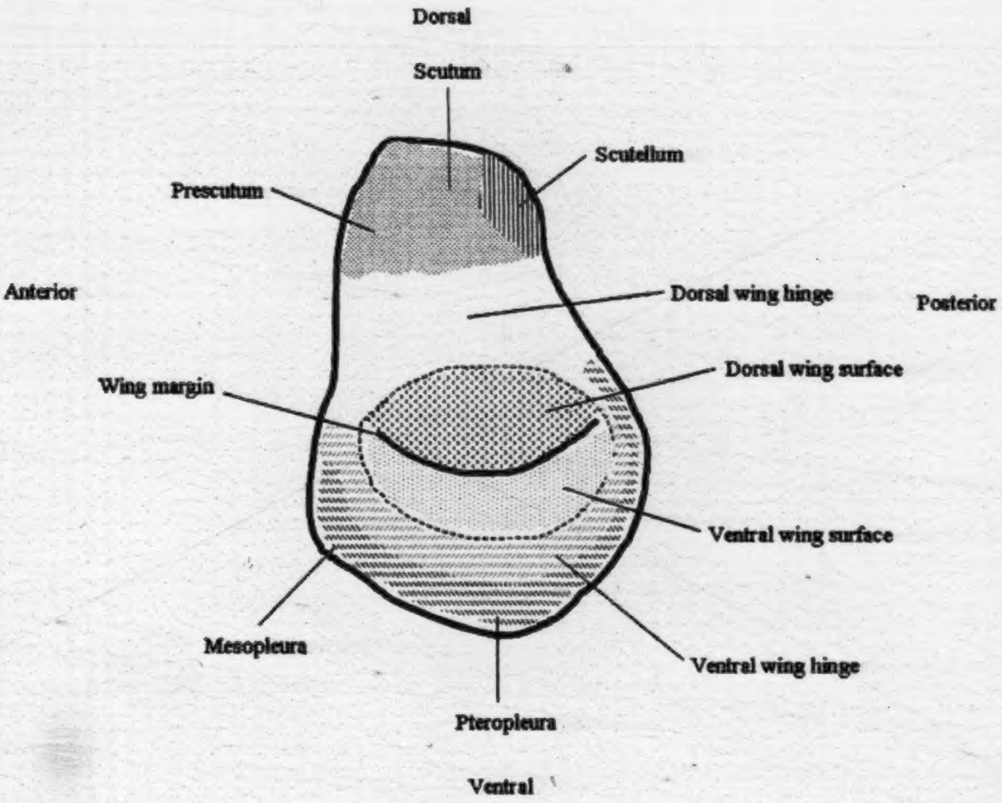

salivary gland imaginal rings
labial disc
dorsal T1 disc
clypeo-labral disc
T1 leg disc
eye-antenna disc
proliferation centres of brain
T2 leg disc
ovary
testis
anterior dorsal histoblast nest
posterior dorsal histoblast nest
genital disc
wing disc
T3 leg disc
haltere disc
foregut imaginal ring
midgut imaginal islands
hindgut imaginal ring
ventral histoblast nest

Figure 1.2: Limb primordia are established by *wg* and *dpp* signaling. *engrailed* (*en*), a homeodomain transcription factor is expressed as a stripe at the posterior of the parasegment boundary (Fjose et al., 1985; Kornberg et al., 1985), while *wg* is expressed as a stripe anterior to the parasegment boundary (Baker, 1987; Cohen et al., 1993). *dpp* is expressed in a lateral stripe perpendicular to the cells expressing *wg* and *en* (St. Johnston and Gelbart, 1987; Cohen et al., 1993). The cells in the vicinity of the intersection between Wg and Dpp become specified as a limb primordium (Cohen et al., 1993).


limb primordium
limb primordium
Dpp
Wg
En
Wg
En

Parasegment boundary

Figure 1.3: Fate map of the wing imaginal disc. Fate map of the third instar wing imaginal disc with the corresponding adult structures labeled (Bryant, 1975). The wing margin corresponds to the D/V boundary in the wing disc. Figure modified from Bryant, 1975.


Dorsal
Scutum
Scutellum
Prescutum
Anterior
Dorsal wing hinge
Posterior
Dorsal wing surface
Wing margin
Ventral wing surface
Mesopleura
Ventral wing hinge
Pteropleura
Ventral

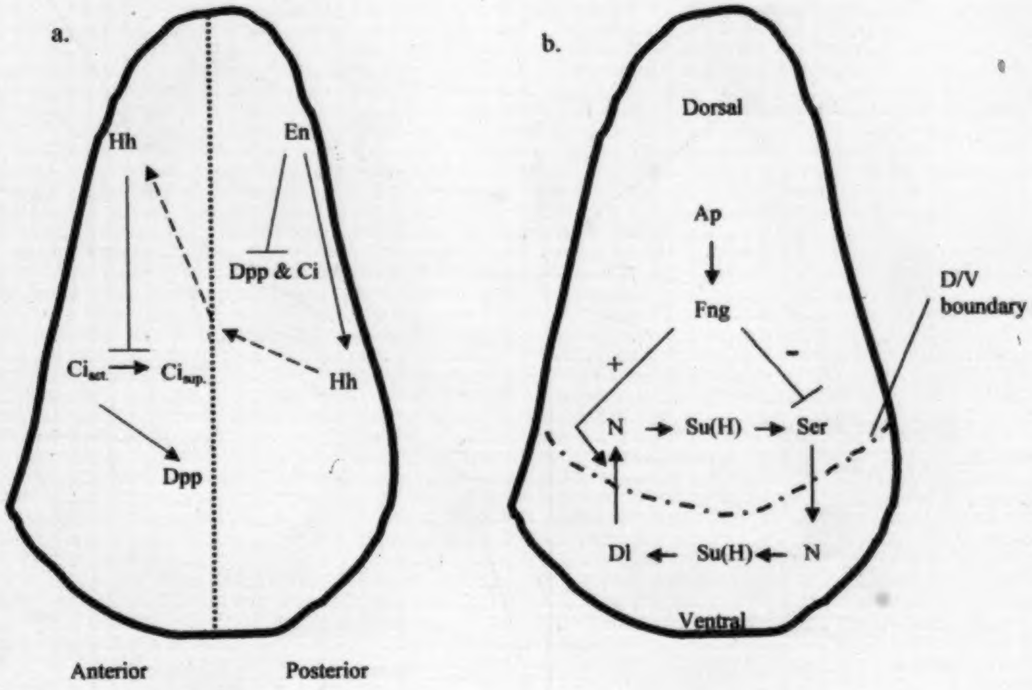
third instar stage, the eye portion of the eye-antennal disc contains about 2000 cells (Becker, 1957). When the larva becomes a pupa, the two eye-antennal discs evaginate and fuse to consequently become the compound eye and head structures (Madhavan and Schneiderman, 1977).

1.3 Wing development

The patterning of the wing disc can be divided into several steps. In the first step, cells are compartmentalized along the A/P axis (Garcia-Bellido, 1976; Wieschaus and Gehring, 1976). This is due to the function of the homeobox gene *engrailed* (*en*) which prevents cells in the anterior compartment from crossing the A/P axis to the posterior compartment and vice versa (Morata and Lawrence, 1975; Kornberg, 1981). *en* is expressed in the posterior compartment of the wing disc and this expression activates *hh* and represses *dpp* (Tabata et al., 1992; Zecca et al., 1996). En renders posterior cells non-responsive to the Hh signal, possibly by repressing the product of the *cubitus interruptus* (*ci*) gene, which is the transcription factor that activates the Hh pathway (Aza-Blanc et al., 1997). However, Hh can migrate across the A/P border to anterior cells where it can prevent cleavage of Ci (Aza-Blanc et al., 1997). Uncleaved Ci is the active form of the protein and is thought to activate genes such as *dpp* and *patched* (*ptc* encodes the receptor for the Hh protein) (Alexandre et al, 1996; Aza-Blanc et al., 1997; Methot and Basler, 1999). The cleaved form, Ci-75, acts as a suppressor and represses activation of genes such as *dpp* (Aza-Blanc et al., 1997; Methot and Basler, 1999). In the anterior compartment, close to the A/P border, where the concentration of the uncleaved form of Ci is the highest and Ci-75 is the lowest (Methot and Basler, 1999), Hh induces the morphogen *dpp* (Basler and Struhl, 1994) which is largely responsible for the patterning of the wing (Zecca et al., 1995; Nellen et al., 1996). A summary of the formation of the A/P axis is found in Figure 1.4a.

The D/V compartment boundary of the wing disc is formed after the A/P boundary is established (Garcia-Bellido et al, 1976; Blair, 1995). The LIM-homeobox gene *apterous* (*ap*) encodes a transcription factor expressed in the dorsal compartment of the wing disc (William et al., 1993; Cohen et al., 1992; Diaz-Benjumea and Cohen, 1993) and is activated in mid second instar wing discs (Williams et al., 1993). The Ap protein activates the gene *dorsal wing* (*dlw*) which is required for dorsal patterning of the wing (Tiong et al., 1995). Ap also activates the product of *fringe* (*fng*), a boundary-specific signaling molecule (Irvine and Wieschaus, 1994) which modulates the activity of Serrate (Ser), and Delta (Dl), which in turn are transmembrane ligands for the receptor Notch (N) (Fehon et al., 1990; Rebay et al.,1991). The Fng protein brings about the differential responsiveness of dorsal and ventral cells to Ser and Dl, such that dorsal cells respond to Dl, resulting in N activation (Fleming et al., 1997; Panin et al., 1997). This leads to activation of downstream target genes, including *Ser* (Panin et al., 1997). In turn, Ser signals back from dorsal to ventral cells activating N, leading to the transcription of downstream genes, including *Dl* (Panin et al., 1997). Fng blocks the ability of Ser to signal to other dorsal cells, such that Ser induces transcription of downstream genes including *Dl* in ventral cells (Fleming et al., 1997; Panin et al., 1997) whereas Fng potentiates Dl signaling only in cells receiving the Dl signal (Panin et al., 1997). Consequently, both Ser and Dl activate *N* expression along the D/V boundary (Panin et al., 1997) as summarized in Figure 1.4b.

Figure 1.4: Formation of the A/P (a) and D/V (b) axis in the wing disc. In panel (a), the green arrow in the posterior compartment represents activation of *hh* by En. The red bar in the posterior compartment represents En suppression of the Hh pathway (En suppresses Ci which is the transcription factor for the Hh pathway) and *dpp*. The dotted black arrow indicates migration of Hh from the posterior to the anterior compartment. In the anterior compartment, the red bar illustrates Hh preventing cleavage of Ci to activate transcription of *dpp* (green arrow). Abbreviations: $\text{Ci}_{\text{act.}}$ is the active form of Ci, while $\text{Ci}_{\text{sup.}}$ is the suppressor form. In panel (b), Ap activates *fng* expression, Fng acts on the two transmembrane ligands Ser and Dl which restricts *N* expression along the D/V boundary marking the future wing margin (Panin et al., 1997). When the N receptor is activated by ligand interaction, the transcription factor *Suppressor of Hairless* (*Su*(*H*)) localizes to the nucleus where it induces transcription of downstream genes (reviewed in Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999). The "+" symbol represents Fng potentiating Dl signaling in dorsal cells and the "-" symbol represents Fng blocking the ability of Ser to signal to dorsal cells.


a.
Hh
En
Dpp & Ci
$Ci_{act.}$
$Ci_{sup.}$
Hh
Dpp
Anterior
Posterior
b.
Dorsal
Ap
Fng
D/V
boundary
+
-
N
Su(H)
Ser
Dl
Su(H)
N
Ventral

The N pathway also activates *wg* at the D/V boundary (Neumann and Cohen, 1996). The Wg protein along with the N pathway, initiates *vg* expression through the *vg* boundary enhancer (*vg*BE) during the second larval instar (Kim et al., 1996; Klein and Arias, 1999; Klein et al., 1998; Williams et al., 1994). Subdivision of the wing field starts with the induction of *vg* (Kim et al., 1996; Williams et al., 1993). N signaling and Vg collaborate to induce the expression of genes that are required for the establishment and patterning of the wing margin along the D/V boundary, such as *wg* (Klein and Arias, 1999; Klein et al., 1998). In turn, stabilization of *vg* expression requires the continued activity of *wg* (Kim et al., 1996; Klein and Arias, 1999). After the establishment of the expression of *wg* and *vg* in the wing primordium, the blade begins to grow (Klein and Arias, 1999). Regions of *wg* expression that fall outside of the domain of *vg* expression will become the future wing hinge (Klein et al., 1998). The expression of *vg* is maintained in the cells of the blade that lie outside of the domains of N signaling through the activity of the *vg* quadrant enhancer (*vg*QE), which drives expression of *vg* in the four quadrants of the wing pouch (Kim et al., 1996; Klein and Arias, 1999). Wg produced at the D/V boundary and Dpp produced at the A/P boundary (as mentioned previously) diffuse from their sources and together with Vg (expressed via *vg*BE) control *vg* expression in the wing pouch through the *vg*QE (Kim et al., 1996; Klein and Arias, 1999; Neumann and Cohen, 1997). Wg and Vg act together in the developing wing blade to activate genes that will control further differentiation of the wing (Klein and Arias, 1999), such as *distalless* (*dll*) which is required for bristles and hairs at the wing margin (Gorfinkiel et al., 1997). Activation of *sd* during early development is also dependent on Vg (William et al., 1993) and expression of *vg* in the wing pouch is required to maintain *sd* expression in the pouch (Paumard-Rigal et al., 1998; Varadarajan and Vijay Raghavan, 1999) (a more detailed regulatory loop between Sd and Vg will be discussed in chapter 4). In addition, Sd and Vg interact for the activation of target genes in the formation of the wings (Halder et al., 1998b; Simmonds et al., 1998). How the Sd-Vg complex functions in wing development will be discussed in the introduction of chapter 3.

In addition to the A/P and D/V axes, the wing may also be divided into the proximal/ distal axis (Blair, 1995; Klein, 2001). The wing disc also forms half the notum (proximal) in addition to forming the wing (distal) (Bryant, 1975). The formation of the notum is dependent on the activity of the EGF-R signaling receptor (Wang et al., 2000). This pathway is activated by the secreted neuregulin-like signaling protein Vein (Vn) (Schnepp et al., 1996; Wang et al., 2000) which is expressed in the dorsal part of the disc during the second larval instar (Simcox et al., 1996; Wang et al., 2000). Vn is restricted to the dorsal part of the wing disc by the suppressive influence of Wg in the ventral region of the disc (Wang et al., 2000). Conversely, *wg* expression is repressed in the dorsal region of the disc by the EGF-R pathway (Baonza et al., 1997; Wang et al., 2000). This antagonistic relationship between the Wg and EGF-R pathways helps to divide the early disc into notum and wing regions (Wang et al., 2000). EGF-R activates the *iroquois* gene complex (*iro-c*) (Wang et al., 2000), a transcription factor which is required for the proper formation of the most proximal dorsal part of the hinge and definition of the border between notum and wing (Diez del Corral et al., 1999).

In addition, two transcription factors encoded by *homothorax* (*hth*) and *teashirt* (*tsh*) are required for the proper development of the hinge (Azpiazu and Morata, 2000;

Casares and Mann, 2000). During the second larval instar stage, both factors are expressed in all cells of the wing discs before becoming restricted to the hinge region during the third larval instar (Azpiazu and Morata, 2000; Casares and Mann, 2000). The expression of *hth* requires Wg and Tsh in the hinge area (Azpiazu and Morata, 2000; Casares and Mann, 2000). The Tsh and Hth proteins collaborate to suppress wing blade formation by repressing the activation of *vg*QE (Azpiazu and Morata, 2000; Casares and Mann, 2000).

1.4 Eye development

Each *Drosophila* compound eye is a honeycomb matrix of about 750 "ommatidial" subunits and each subunit has eight photoreceptors or R cells (R1-R8) (Ready et al., 1976). Each photoreceptor contains a light sensing organelle called a rhabdomere with the overall arrangement of the photoreceptors forming a trapezoidal pattern (Ready et al., 1976). Photoreceptors R1-R6 surround the rhabdomeres of the R7 and R8 cells with R8 sitting directly underneath R7 (Ready et al., 1976). Above the photoreceptors, each adult ommatidium has four "cone" cells that secrete the lens (Ready et al., 1976; Tomlinson et al., 1987a). Surrounding the cones cells are 1^0, 2^0, and 3^0 pigment cells and bristle cells (Ready et al., 1976). See Figure 1.5 for a diagram of the compound eye.

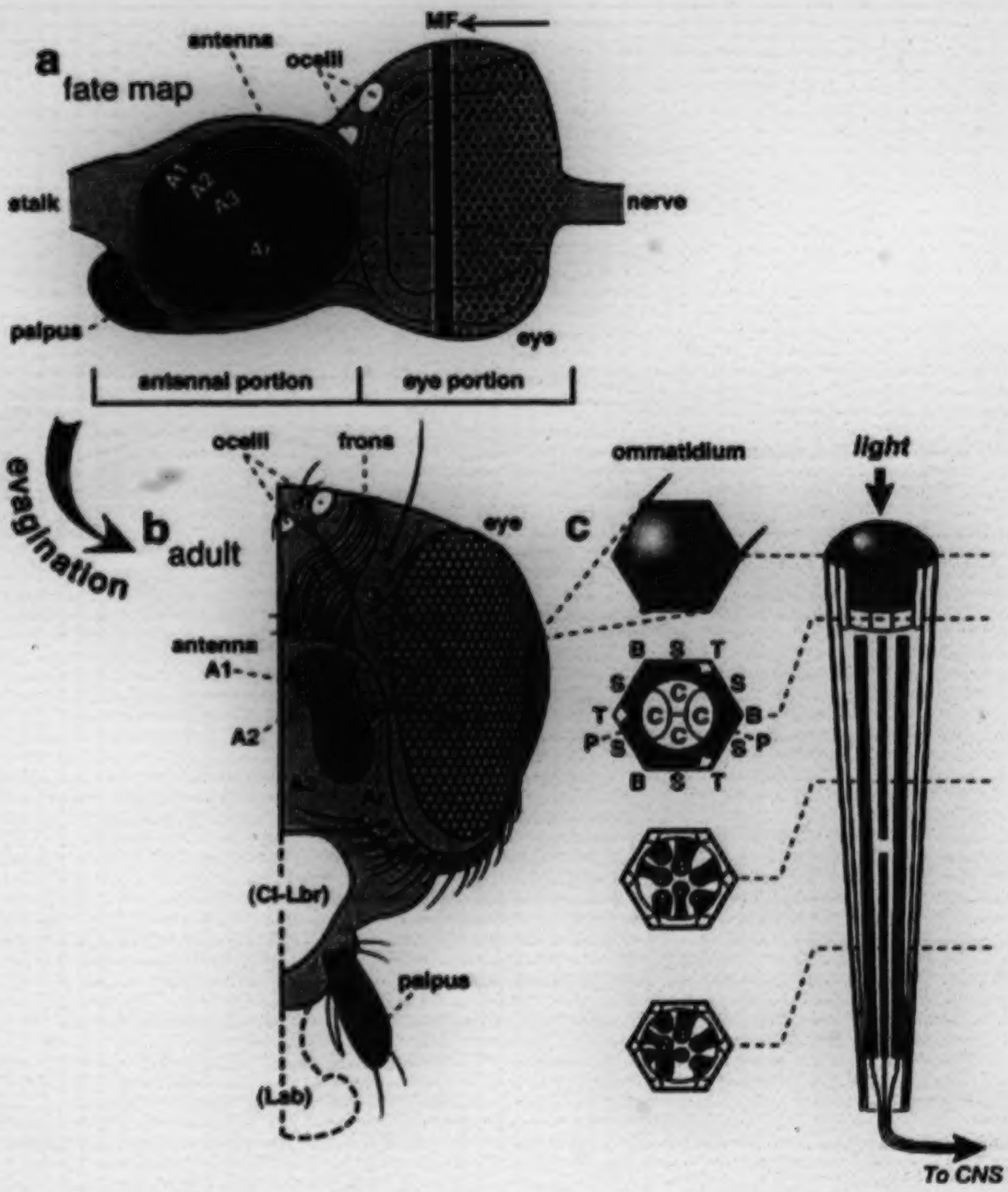
The *Drosophila* compound eye develops from the eye-antennal imaginal disc (Figure 1.5) (Garcia-Bellido and Merriam, 1969; Wieschaus and Gehring, 1976). Eye development is a complex process involving several important steps; the first is the

Figure 1.5: Fate map of the eye-antenna disc and structure of the ommatidia. a) Fate map of a mature eye-antenna disc with the corresponding structures in b. Abbreviations: A1 to A3 are antennal segments 1 to 3. Ar (Arista), MF (morphogenetic furrow as stated in the text) is represented by the black bar, arrow is the direction of the MF movement, and dots symbolize photoreceptor clusters.

b) Half of a head. The frons manifest parallel grooves as human finger prints (Ferris, 1950; Hodgkin and Bryant, 1978) The ocelli are used for detecting moving shadows (Benzer, 1991). The third antennal segment senses odors (Kim et al., 1998). The arista appear to sense temperature and humidity (Foelix et al., 1989). Interommatidial bristles are omitted. Abbreviations: Cl-Lbr (clypeus and labrum forming from the clypeolabral disc), Lab (labellum derives from the labial disc). These structures are blank since they are not formed from the eye-antennal disc. The other half of the head will be formed by the other eye-antennal disc.

c) Structure of a single ommatidium. At the right is the view of the entire conical ommatidium. Cross sections of these structures are on the left shown in three levels. Abbreviations: B (bristle), C (cone cell), P, S, T are primary, secondary, tertiary pigment cells respectively, 1 to 8 are photoreceptors R1 to R8. The four cone cells are surrounded by two primary cells, bordered by six secondary cells, with three bristles and three tertiary cells at alternating vertices.

(Images obtained and modified from flybase at http://flybase.bio.indiana.edu/)


a
fate map
antenna
ocelli
MF
stalk
A1
A2
A3
Ar
nerve
palpus
eye
antennal portion
eye portion
evagination
b
adult
ocelli
frons
eye
antenna
A1
A2
(Cl-Lbr)
palpus
(Lab)
c
ommatidium
light
B S T
S S
T C C C C B
P S S P
B S T
To CNS

specification of the eye imaginal disc during embryonic life. Some key genes involved include *eyeless* (*ey*), *twin of eyeless* (*toy*), *sine oculis* (*so*), *eye absent* (*eya*), *eye gone* (*eyg*), and *dachshund* (*dac*) (Bonini et al., 1993; Cheyette et al., 1994; Czerny et al., 1999; Halder et al., 1995; Halder et al., 1998a; Jang et al., 2003; Jun et al., 1998; Mardon et al., 1994). *ey*, *toy*, and *eyg* encode Pax-like proteins, and in the mammalian system the *Pax* genes are also required for eye development (Czerny et al., 1999; Jun et al., 1998; Quiring et al., 1994). *so*, *eya*, and *dac* encode evolutionarily conserved nuclear proteins that are also essential for *Drosophila* eye development (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994). It is known that *toy* acts upstream of *ey* to activate its expression (Czerny et al., 1999), and during embryonic development, *ey*, *eyg* and *so* are expressed to define the progenitor regions of the optic lobe and eye disc (Pignoni et al., 1997). The eye disc grows during early larval stages, and in the middle of the third larval instar stage, a wave of photoreceptor differentiation initiates at the posterior edge of the disc (Ready et al., 1976). This wave of differentiation is marked by an apical constriction of the disc epithelium known as the morphogenetic furrow (MF) (Ready et al., 1976). Cells located posterior to the MF assemble gradually into ommatidial clusters, while cells located anteriorly are unpatterned and divide actively (Ready et al., 1976; Tomlinson and Ready, 1987b). Each ommatidium is assembled such that R8 is the initial photoreceptor in the "ommatidial" subunit (Tomlinson and Ready, 1987b). The remaining seven photoreceptors and four lens producing cone cells are recruited during the late third instar (Tomlinson and Ready, 1978b). During pupal development, the pigment and bristle cells join the ommatidium at the periphery and any surplus undifferentiated cells are removed by apoptosis (Wolff and Ready, 1993).

MF initiation requires *eya* and *so* (Chen et al., 1997). It is thought that Eya and So form a complex with So providing the DNA binding component and Eya providing the transcription activation component of the complex (Chen et al., 1997; Pignoni et al., 1997). In addition, it is hypothesized that Dac may associate with an unknown basal transcription activator forming a complex which interacts with So-Eya (Chen et al., 1997). This interaction is mediated by physical association between Dac and Eya, and may provide specificity to the Eya-So complex to activate transcription of MF initiation essential genes (Chen et al., 1997). At the intermediate stage of eye development, another level of MF initiation control is provided by the action of Dpp and Hh (Dominguez and Hafen, 1997; Royet et al., 1997). At this point, Dpp and Hh are present at the posterior of the MF with Hh directly regulating MF initiation and propagation while Dpp is required to repress *wg* which prevents ommatidial differentiation (Dominguez and Hafen, 1997; Royet et al., 1997). Again, the So-Eya complex is also necessary for MF progression, while Dac is not required for this process (Chen et al., 1997; Pignoni et al., 1997). It has been hypothesized that other unknown factors may provide the specificity for So-Eya to activate genes necessary for MF progression (Chen et al., 1997). Another role So-Eya plays in eye development is the differentiation of photoreceptors (Pignoni et al., 1997). It has been suggested that *ey* also plays a role in this process by directly controlling rhodopsin gene expression (Sheng et al., 1997). Therefore, it appears that the So-Eya complex controls different steps in eye development with various transcriptional activators (Pignoni et al., 1997).

1.5 Eukaryotic Transcription

In general in an eukaryotic cell, most genes are repressed except for the "housekeeping" genes. Transcription of other genes occurs only in response to specific physiological needs of the cell. Condensation of DNA with histone octamers into nucleosomes (chromatin) (Kornberg, 1974) prevents access of the general transcription machinery and other co-activators to the DNA and thereby preventing activation of transcription (Han and Grunstein, 1998; Knezetic and Luse, 1986; Lorch et al., 1987). Therefore, the initiation of transcription involves relieving the level of condensation of the chromatin followed by interaction of RNA polymerase and accessory factors with the promoter. Histone acetyltransferase is known to acetylate histones to relieve transcription repression (Allfrey, 1977; Brownell et al., 1996; Kuo et al., 1998). Furthermore, chromatin remodeling complexes also "remodel" the structure of the chromatin in an ATP-dependent manner to allow for transcription (Cairns et al., 1996; Cote et al., 1994). In *Drosophila*, this complex is known as the NURF complex (Georgel et al., 1997).

RNA polymerase II (RNAPII) is responsible for all mRNA synthesis (Chambon, 1975), and is a large multi-protein complex requiring general transcription factors (GTFs) to initiate transcription (Matsui et al.,1980; Davison et al., 1983). The GTFs for RNAPII are TFIIB, -D, -E, -F, and –H (reviewed in Conaway and Conaway, 1997). TFIIB aligns the RNAPII properly on the DNA template by interacting with the TATA box sequence via the TATA box binding proteins (TBPs) (Li et al., 1994; Leuther et al., 1996). TFIID is responsible for promoter recognition (Shen et al., 1997). TFIIE suppresses transcriptional arrest during the early stages of transcription elongation (Dvir et al., 1996;

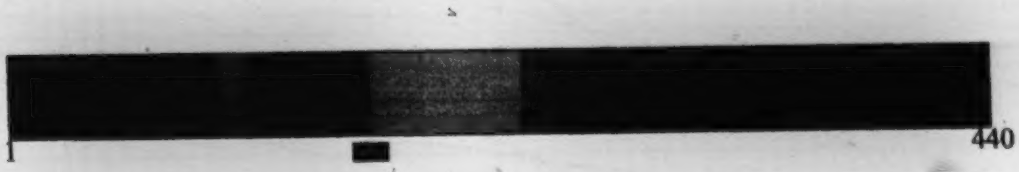
Dvir et al., 1997; Kugal and Goodrich, 1998; Kumar et al., 1998). TFIIF suppresses pauses of the RNAPII and also facilitates in unwinding of DNA around the polymerase (Robert et al., 1998; Price et al., 1989). Finally, TFIIH includes an ATP dependent helicase that unwinds the promoter around the start site to trigger the initiation of transcription (Roy et al., 1994; Schaeffer et al., 1993). Another factor necessary for transcription is the mediator which was discovered to interact with the C-terminal domain of RNAPII to form the "holoenzyme complex" (Thompson et al., 1993; Kim et al., 1994). The mediator is thought to interact with activators bound to enhancer elements to transduce regulatory information from the enhancer to the promoter (Bjorklund and Kim, 1996).

In addition to GTFs, there are transcription factors for the activation of specific genes in a temporal and spatial manner for the development of an organism. These transcription factors have to recognize and bind specific DNA sequences and they also have to contain a transcription activation component. What makes Sd different from these transcription factors is that Sd contains a DNA binding domain, but no known activating domain. In the wing tissue, activation is accomplished by the ability of Sd to interact with Vg, which serves as the activating component of the Sd-Vg complex. In the eye, however, it is unclear if Sd functions in a similar manner.

1.6 *scalloped* (*sd*)

The *sd* gene was cloned by transposon tagging with a P[ry^+] insertion mutation and chromosome walking (Campbell et al., 1991). The *sd* locus is located at 1-51.5/13F (Gruneberg, 1929). *sd* has at least three transcript classes that are presumed to arise by alternative RNA splicing of a 12kb primary transcription unit (Campbell et al., 1992). One of these transcripts (E21) has 12 exons and encodes a 440 amino acids protein containing an evolutionarily conserved DNA binding motif, the TEA domain (Campbell et al., 1992). The TEA DNA binding domain is found in other nuclear regulatory proteins such as the yeast trans-acting factor Tec-1, the Aspergillus *abaA* regulatory gene product ABAA, and the human transcription enhancer factor-1 (TEF-1) (Burglin, 1991). Hence, the name "TEA domain" originates from TE for TEF-1/TEC1, and A for ABAA (Burglin, 1991). The TEA domain of *sd* is most closely related to that of TEF-1, being 98% identical in the TEA DNA binding domain at the amino acid level and 68% identical at the amino acid level over the entire sequence (Campbell et al., 1992). From sequence alignment with the TEF-1 sequence, the TEA DNA binding domain in Sd contains three helices (Burglin, 1991; Campbell et al., 1992). The first, second and third helices include amino acids 95 to 108, 127 to 138, and 147 to 155, respectively, (Burglin, 1991; Campbell et al., 1992). The TEA domain of *Drosophila* Sd extends from amino acids 88 to 159 of the protein and was discovered by sequence alignment with the TEF-1 sequence (Campbell et al., 1992). The putative nuclear localization signal (NLS) of *sd* extends from residue 144 to 163 of the protein (Srivastava et al., 2004). The Vg interacting domain (VID) of Sd is loosely localized to amino acids 220 to 344 of Sd and was determined by sequence alignment with the region of TEF-1 that interacts with a human homologue of Vg (Vg like-1) (Vaudin et al., 1999). There is also a putative finger motif localized to amino acids 416 to 433 of Sd as deduced from sequence alignment with TEF-1 (Campbell et al., 1992). Whether this finger motif is functional is unknown (Campbell et al., 1992). A summary of the known regions of Sd is in Figure 1.6.

Figure 1.6: A diagrammatic representation of the Sd protein motifs. Each domain is color coded with its respective location in the protein. The TEA DNA binding domain contains three helices (Burglin, 1991). The black bar under the protein represents the putative NLS that overlaps the third helix of the TEA domain and the linker domain (Burglin, 1991; Campbell et al., 1992; Srivastava et al., 2004). The linker domain is given its term for convenience. As made clear in later chapters, this domain actually functions in *Drosophila* development. The amino acid residues of the protein go from the amino terminal 1 to the carboxyl terminal 440. Diagram is not drawn to scale.



N-terminal domain (1-87)
C-terminal domain (345-440)

TEA (88-159)
Helix 1 (96-108)
Helix 2 (127-138)
Helix 3 (147-155)

Putative nls (144-163)
VID (220-344)
Linker (160-219)-
Putative finger motif (416-433)

sd is expressed in all imaginal discs in a third instar larva except for the labial disc (Campbell et al., 1992). To be more specific, *sd* expression in a third instar wing disc is concentrated in the areas that will give rise to the wing surface, scutellum, and mesopleura (Figure 1.3) (Campbell et al., 1992), which coincides with the domain of *vg* expression at the same developmental stage (Williams et al., 1993). In the eye-antennal disc, *sd* is expressed immediately behind the MF as it moves across the developing eye disc (Campbell et al., 1992). In addition, *sd* is also expressed in the optic lobes, discrete cells of the cerebral hemispheres lying outside of the optic lobes, and in specific cells of the ventral nerve cord (Campbell et al., 1992).

There are both viable and lethal mutant alleles of *sd* (Campbell et al., 1991). Among the viable alleles are sd^{ETX4} and sd^{58d} (Anand et al., 1990; Campbell et al., 1992). sd^{58d} is γ-ray induced (Ives, 1961) and sd^{ETX4} is a P-element insertion allele (Anand et al., 1990). Both of these alleles cause defects in the wings, supporting a role for Sd in wing development (Anand et al., 1990; Campbell et al., 1992; Campbell et al., 1991). The *sd* lethal alleles include sd^{3L}, sd^{47M}, sd^{11L}, sd^{68L}, and sd^{31H}. They can be subdivided into early and late classes. The early lethal alleles (sd^{3L} and sd^{47M}) die as first instar larvae (Campbell et al., 1991; Srivastava et al., 2004). The late lethals (sd^{11L}, sd^{68L}, and sd^{31H}) survive through the larval instars and die at the pupal stage (Campbell et al., 1991). The phenotypes associated with these alleles suggest that Sd functions in larval and pupal development and has a vital role in the viability of the fly. Interestingly, *sd* lethal alleles sd^{3L}, sd^{47M}, sd^{31H}, and sd^{68L} cannot complement the wing phenotype associated with sd^{ETX4} while sd^{11L} can (Srivastava et al., 2004). Therefore, mutations in certain regions of *sd* are lethal to the fly and yet maintain an ability for normal wing development (sd^{11L}), while mutations in other regions of the gene will cause a wing phenotype and have no effect on the viability of the organism (sd^{ETX4} and sd^{58d}). This suggests that different domains of Sd are important for its role in different developmental processes.

The expression of *sd* in other third instar imaginal discs suggests that it may function in these tissues as well. However, the different domains of Sd important for its various roles in development have not been identified. The different domains of Sd including the TEA DNA binding domain, VID, and finger motif are inferred by sequence alignment with TEF-1 or by *in vitro* experimentation. No experiments have been done *in vivo* to study the functionality of these domains.

Chapter 2: Materials and Methods

2.1 *Drosophila* stocks:

The origins of the various stocks are listed in Table 2.1. All crosses and stocks were raised at room temperature (22^0C to 23^0C) unless stated otherwise.

2.2 Sd deletion constructs generated and the nomenclature system

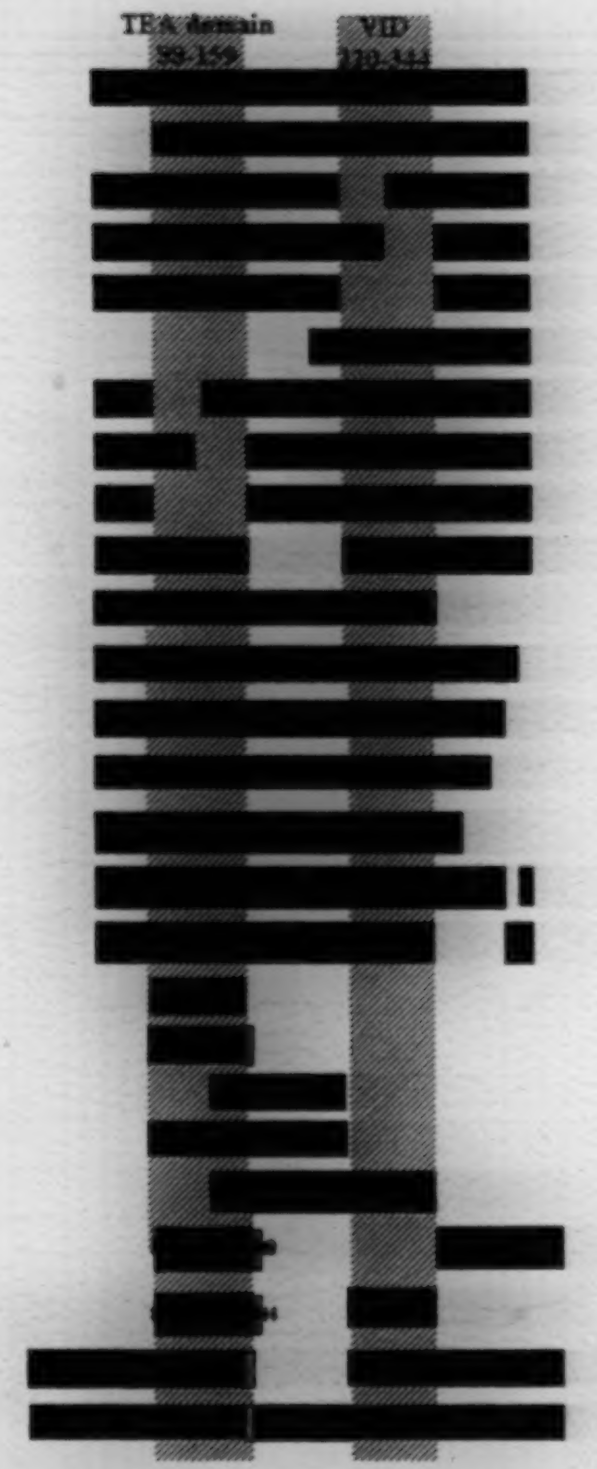
All deletion constructs generated are shown in Figure 2.1 and the nomenclature used for these constructs is as follows. A delta sign (Δ) preceding any deletion construct indicates the encoded amino acids that have been deleted from the wildtype full length protein. Appropriate start or stop codons were engineered into constructs with deletions in the N-terminal or C-terminal ends, respectively. When a deletion construct is presented without a Δ symbol, this illustrates the amino acids that are retained with respect to the full length protein. There are some more complicated constructs that were made by fusing different regions of the *sd* gene. The regions of the Sd protein that are fused in this manner will be designated by the amino acids that are retained and a "+" symbol to indicate the regions that are fused together in frame. Finally, there is one construct that had DNA encoding amino acids 168 to 219 (linker) of Sd replaced with a piece of DNA of equal length from the pBSII(SK+) plasmid. This construct will be referred as Δ168-219R (replacement).

More than one independent transgenic line was generated for the majority of the deletion constructs. Consequently, to distinguish independent transgenic lines of the same construct from each other, each different transgenic line will be identified with a numerical symbol. For example, Δ88-123 $^{\#}$2-2, $^{\#}$2-8 and $^{\#}$3-1 represent three independent

Table 2.1 Summary of stocks used in this study and their respective origins

Allele	Source
sd^{ETX4}	Anand et al., 1990
sd^{58d}	Campbell et al., 1991
vgGal4	Hoffman and Monimura (unpublished)
*ptc*Gal4	Bloomington Stock Centre
yw; *eyeless*Gal4 UAS-Flp;GMR-hid FRT82B/TM2	Bloomington Stock Centre
yw	Bloomington Stock Centre

Figure 2.1: A schematic diagram of all *sd* deletion constructs generated for the rescue assay. The solid black bar indicates the regions of Sd that are retained and open spaces represents regions of Sd that have been deleted. The yellow bar corresponds to eight additional amino acids that extend from the TEA DNA binding domain to include the entire putative NLS (NLS is localized to residues 144 to 163 of Sd) (Srivastava et al., 2004) whereas the pink bar represents the linker region that was replaced with a piece of DNA from pBSII(SK+) of equal length. The designation of each construct is also written on the construct and the locations of the TEA DNA binding domain and the VID are also indicated.


TEA domain
38-159
VID
310-344

transgenic lines of Δ88-123. An explanation of the nomenclature for Δ88-159, and Δ160-219 blunt (b)/not blunt (nb) is described later.

2.3 General outline of how all the *sd* constructs were generated

The procedure to generate each of the *sd* deletion constructs is similar and follows the general steps described below, unless otherwise specified. A list of primer sequences used and their respective T_m values are in Table 2.2. The open reading frame (ORF) of *sd* is illustrated in Figure 2.2.

For constructs with a deletion at the N-terminal domain (5'), the C-terminal domain (3'), or the 5' and 3' termini, the general design for the 5'end primers contained a *Bam*HI site and an ATG start codon (arrow indicates direction of the primer):

BamH1-ATG ⟶

The 3' end primers contained an appropriate restriction site (RS) for cloning and a TAG stop codon:

⟵ ATC-RS

For constructs with internal deletions, the primers were designed with a *Spe*I site at the end of each primer.

The 5'end primers had the following general design:

⟵ *Spe*I

Table 2.2a Primers utilized to generate *sd* deletions and corresponding T_m values

Construct	Primer set	Primer sequence 5' → 3'	T_m (°C)
Δ1-87	5'Δ87	GGGGATCCATGGATGCCGAAGGTGTAT	84
	3' end full	CCTCTAGAATGCAGCTTTTGCTAT	68
Δ1-200	5'Δ-200	GGGGATCCATGAAAACGTCGACTGCGGT	90
	3' end full	CCTCTAGAATGCAGCTTTTGCTAT	68
Δ88-123	Δ88-123a	CCTCACTAGTAGCGGATGACAAGTCCT	82
	Δ88-123b	GAGCACTAGTATGTACGGTCGCAACGA	82
Δ124-159	Δ124-159a	GCAGTACTAGTTTTACCCTCGTCGGATA	82
	Δ124-159b	GCACTAGTCTCCGCGAGATCCAGGC	82
Δ88-159 not blunt	Δ88-159a	CCTCACTAGTAGCGGATGACAAGTCCT	82
	Δ88-159b	GCACTAGTCTCCGCGAGATCCAGGC	82
Δ88-159 blunt	Δ88-159 Blunt(a)	AGCGGATGACAAGTCCTTTTCGT	68
	Δ88-159 Blunt(b)	CTCCGCGAGATCCAGGCGAAA	68
Δ160-219 not blunt	Δ160-219a	GCCACTAGTTTTACGGCGAGCCAGCA	82
	Δ160-219b	GCCACTAGTTGGGAAGGACGAGCCAT	82
Δ160-219 blunt	Δ160-219 Blunt(a)	TTTACGGCGAGCCAGCACTTGG	70
	Δ160-219 Blunt(b)	TGGGAAGGACGAGCCATTGCCA	70
Δ434-440	Sd-full-1	GGGGATCCATGAAAAACATCACCAGCT	80
	3'Δ7	AAGGTACCCTAGTGATGGGTGGTGCCG	86
Δ220-281	Δ220-344a	AGACTAGTGGGCAATTGTGAGGGCG	78
	Δ220-281b	CTTTACTAGTTCTGGGGGCCTTAAAGA	78
Δ282-344	Δ282-344a	AGCCACTAGTTTTCTCCGGAAACTTGT	78
	Δ220-344b	GGGACTAGTAAGCAAGTGGTGGAGAA	78
Δ220-344	Δ220-344a	AGACTAGTGGGCAATTGTGAGGGCG	78
	Δ220-344b	GGGACTAGTAAGCAAGTGGTGGAGAA	78
Δ345-440	Sd-full-1	GGGGATCCATGAAAAACATCACCAGCT	80
	3'Δ 96	AATCTAGACTAGCCAAAGGAGCAAACGA	80
Δ365-440	Sd-full-1	GGGGATCCATGAAAAACATCACCAGCT	80
	3'Δ76	AAGGTACCCTAGACGTAGCGATTGTTC	80
Δ391-440	Sd-full-1	GGGGATCCATGAAAAACATCACCAGCT	80
	3'Δ50	AAGGTACCCTAATAGCGTTCCGGTAGG	82
Δ416-440	Sd-full-1	GGGGATCCATGAAAAACATCACCAGCT	80
	3'Δ25	AAGGTACCCTACAACAGTGTCTCCTGC	82
Δ345-415	Δ345-415a	GCCAAAGGAGCAAACGATTGT	60
	Δ345-415b	TGCATAGCCTATGTGTTTGAGGT	60

Table 2.2b Primers utilized to generate *sd* deletions and corresponding T_m values

Construct	Primer set	Primer sequence 5' ⟶ 3'	T_m (°C)
Δ416-433	Δ416-433a	CAACAGTGTCTCCTGCGTCTCG	62
	Δ416-433b	ATATACCGTCTAATTAAGGAATAG	62
Δ168-219	Δ168-219a	CCGACTAGTGATTTTCGCCTGGATCTC	82
	Δ160-219b	GCCACTAGTTGGGAAGGACGAGCCAT	82
88-159	88-159a	CGCGGATCCGATGCCGAAGGTGTATG	84
	88-159b	AAGGTACCCTATTTACGGCGAGCCAGC	84
88-167	88-159a	CGCGGATCCGATGCCGAAGGTGTATG	84
	88-167b	TCTGGTACCCTAGATTTTCGCCTGGATC	84
137-219	137-219a	TTGGATCCATGCTGCGCACAGGCAAAA	86
	137-219b	ACTCTAGACTAGGGCAATTGTGAGGGCG	86
88-219	5'Δ87	GGGGATCCATGGATGCCGAAGGTGTAT	84
	137-219b	ACTCTAGACTAGGGCAATTGTGAGGGCG	86
137-344	137-219a	TTGGATCCATGCTGCGCACAGGCAAAA	86
	137-344b	CCTCTAGACTAGCCAAAGGAGCAAACGAT	86
137-219 +345-440	137-219a	TTGGATCCATGCTGCGCACAGGCAAAA	86
	137-219+345-440b	CGCTCTAGACTATTCCTTAATTAGACGG	80
	Template: Δ220-344-pUAST		
88-167+ 345-440	Δ168-219a	CCGACTAGTGATTTTCGCCTGGATCTC	82
	Δ220-344b	GGGACTAGTAAGCAAGTGGTGGAGAA	78
	Template: Δ1-87 pBSII(SK+)-2xFLAG		
88-167+ 220-344	5'Δ87	GGGGATCCATGGATGCCGAAGGTGTAT	84
	137-344b	CCTCTAGACTAGCCAAAGGAGCAAACGAT	86
	Template: Δ168-219-pUAST		

The red, blue, purple, and brown bases represent the *Bam*HI, *Xba*I, *Spe*I, and *Kpn*I restriction sites, respectively. The green and orange bases indicate the start and stop codons, respectively. The template used to generate the corresponding *sd* constructs was full length *sd* ORF in pUC19 unless otherwise stated. For construction of 88-167+345-440, DNA encoding amino acids 88 to 440 of *sd* were cloned into pBSII(SK+)-2xFLAG with *Bam*HI and *Xba*I to serve as the template.

Figure 2.2 a and b: The *sd* ORF (a) and amino acid composition of the protein (b). In a, the orange bases indicate the start codon, the red and green colors represent the TEA DNA binding domain and the VID, respectively, as defined (Campbell et al., 1992; Vaudin et al., 1999). The blue bases denote the stop codon. The bases following the stop codon were sequences that had been cloned into pET16b along with the *sd* ORF. The underlined region represents the putative NLS. In b, the red and green amino acids represent the TEA DNA binding domain and the VID, respectively. The underlined region represents the putative NLS.

a. 1 ATGAAAAACA TCACCAGCTC GAGCACTTGC AGCACTGGGC TGCTGCAATT GCAGAACAAC
61 CTGAGCTGCA GCGAGTTGGA AGTTGCCGAG AAGACAGAAC AACAGGCAGT TGGACCCGGC
121 ACCATACCAT CACCGTGGAC ACCAGTGAAT GCCGGTCCTC CAGGCGCACT TGGATCGGCA
181 GACACAAATG GCAGCATGGT GGATAGCAAA AACCTGGATG TCGGTGATAT GAGCGATGAC
241 GAAAAGGACT TGTCATCCGC TGATGCCGAA GGTGTATGGA GTCCAGATAT CGAGCAGAGC
301 TTTCAAGAGG CTTTATCTAT ATATCCGCCG TGCGGACGTA GAAAAATCAT TTTATCCGAC
361 GAGGGTAAAA TGTACGGTCG CAACGAGCTA ATCGCACGAT ATATAAAACT GCGCACAGGC
421 AAAACGAGAA CCAGGAAGCA AGTCAGTTCG CACATCCAAG TGCTGGCTCG CCGTAAACTC
481 CGCGAGATCC AGGCGAAAAT CAAAGTGCAA TTCTGGCAAC CTGGACTACA GCCAAGCACG
541 TCCCAAGATT TCTATGATTA CAGCATCAAG CCCTTCCCCC AGCCGCCGTA TCCAGCTGGC
601 AAAACGTCGA CTGCGGTTTC CGGGGACGAA ACTGGAATTC CGCCCTCACA ATTGCCCTGG
661 GAAGGACGAG CCATTGCCAC GCACAAATTC CGCTTACTCG AGTTTACGGC GTTCATGGAA
721 ATCCAGAGAG ATGAAATTTA TCACCGGCAT CTATTCGTTC AACTTGGCGG CAAGCCATCC
781 TTTTCCGATC CATTGCTTGA GACTGTTGAT ATACGGCAAA TATTCGACAA GTTTCCGGAG
841 AAATCTGGGG GCCTTAAAGA TCTCTACGAA AAGGGTCCAC AGAATGCGTT TTACCTAGTT
901 AAATGCTGGG CGGACCTGAA TACCGATCTA ACAACCGGCA GCGAAACGGG TGATTTCTAT
961 GGCGTAACCA GCCAATACGA AAGCAACGAG AATGTCGTGC TCGTGTGCTC CACAATCGTT
1021 TGCTCCTTTG GCAAGCAAGT GGTGGAGAAG GTGGAAAGCG AGTACTCCCG ACTGGAGAAC
1081 AATCGCTACG TCTATCGCAT TCAACGCTCC CCCATGTGCG AGTACATGAT CAACTTTATT
1141 CAGAAGCTGA AGAACCTACC GGAACGCTAT ATGATGAACA GTGTGCTGGA AAACTTTACA
1201 ATATTGCAAG TAATGAGGGC CCGCGAGACG CAGGAGACAC TGTTGTGCAT AGCCTATGTG
1261 TTTGAGGTGG CGGCCCAGAA CAGCGGCACC ACCCATCACA TATACCGTCT AATTAAGGAA
1321 TAGCAAAAGC TGCAT

b. 1 MKNITSSSTC STGLLQLQNN LSCSELEVAE KTEQQAVGPG TIPSPWTPVN AGPPGALGSA
61 DTNGSMVDSK NLDVGDMSDD EKDLSSADAE GVWSPDIEQS FQEALSIYPP CGRRKIILSD
121 EGKMYGRNEL IARYIKLRTG KTRTRKQVSS HIQVLARRKL REIQAKIKVQ FWQPGLQPST
181 SQDFYDYSIK PFPQPPYPAG KTSTAVSGDE TGIPPSQLPW EGRAIATHKF RLLEFTAFME
241 IQRDEIYHRH LFVQLGGKPS FSDPLLETVD IRQIFDKFPE KSGGLKDLYE KGPQNAFYLV
301 KCWADLNTDL TTGSETGDFY GVTSQYESNE NVVLVCSTIV CSFGKQVVEK VESEYSRLEN
361 NRYVYRIQRS PMCEYMINFI QKLKNLPERY MMNSVLENFT ILQVMRARET QETLLCIAYV
421 FEVAAQNSGT THHIYRLIKE

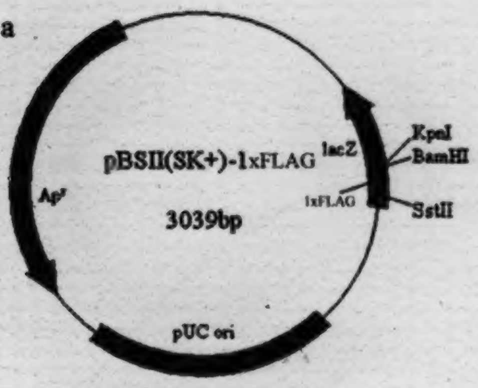
The 3' end primers had the following general design:

*Spe*I ⟶

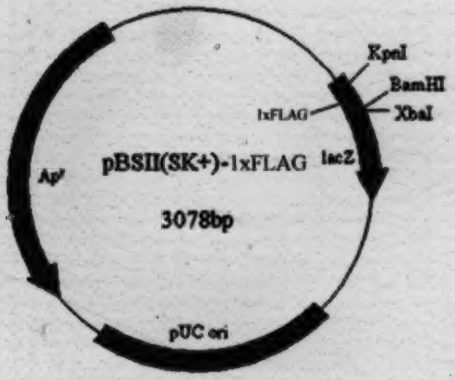
Once the desired *sd* fragments were amplified via PCR or inverse PCR with the appropriate primer set, they were subjected to electrophoresis in a 1% TAE agarose gel. Unless stated otherwise, 1% agarose gels were used to electrophorese all samples. The desired bands were isolated from the gel and purified using the Qiaquick Gel Extraction Kit and digested with the appropriate enzyme(s) for ligation into the injection vector pUAST (Brand and Perrimon, 1993) or the purified fragments were subcloned into the pBSII(SK+)-1xFLAG or pBSII(SK+)-2xFLAG plasmid (Figure 2.3) before ligation into pUAST. For any *sd* internal deletion constructs generated by inverse PCR, there was an additional step to re-circularize the PCR products after they had been isolated from the gel. Once the desired *sd* insert was ligated into the appropriate vector, 25% of the ligation reaction was then transformed into competent DH5α *E. coli* (cDH5α) cells. Bacterial colonies were selected and grown in 5mL LB media with Ampicillin. Plasmid DNA was then isolated from these cultures using a Qiaprep Mini Prep kit and digested with the appropriate enzyme(s) to test for the presence and/or orientation of the insert. If the *sd* deletion insert was in another vector and not pUAST, restriction digestion would be performed with the appropriate enzyme(s) to remove the insert from the vector before ligation into pUAST. Following the isolation of a bacterial colony with the desired *sd* deletion cloned into pUAST, it was digested with *Eco*RV and *Pst*I, unless otherwise stated, to determine the presence and/or orientation of the insert in pUAST. Correct constructs were amplified via the Qiagen Maxi Prep kit. Restriction

Figure 2.3: Diagram of the pBSII(SK+)-1xFLAG and pBSII(SK+)-2xFLAG plasmids. Panel (a) shows pBSII(SK+)-1xFLAG plasmid for adding a 1xFLAG to the following *sd* constructs: Δ 434-440, Δ 416-440, Δ 391-440, Δ 365-440, 88-159, 88-167, Δ168-219, and Δ168-219R. Panel (b) shows pBSII(SK+)-2xFLAG plasmid for adding a 2xFLAG to the following *sd* constructs: 137-219, 88-219, 137-344, Δ345-415, Δ416-433, 137-219+345-440, 88-167+220-344, and 88-167+345-440. Abbreviations are as follows: Ap^r; Ampicillin resistance, pUC ori; pUC19 origin of replication.

a


KpnI
BamHI
lacZ
pBSII(SK+)-1xFLAG
1xFLAG
SstII
Ap^r
3039bp
pUC ori

b


KpnI
BamHI
1xFLAG
XbaI
pBSII(SK+)-1xFLAG
lacZ
Ap^r
3078bp
pUC ori

digestion and spectrophotometer readings were carried out to ascertain the concentration and purity of the sample. Finally, the plasmid was sequenced to ensure that the *sd* ORF was correct. Once this was determined, the plasmid would be further purified using ethanol precipitation steps and treated with the helper plasmid Δ2-3 (a transposase source) before micro-injection into *yw* embryos. Details of the restriction digest, ligation, sequencing reactions, transformation into cDH5α, Qiaquick Gel Extraction, Qiaprep Mini Prep, Qiagen Maxi Prep, ethanol precipitation, Δ2-3 precipitation, and the micro-injection processes will all be described later.

2.4 Construction of full length *sd* in pUC19 (*sd*pUC19)

Full length *sd* in puc19 was constructed to serve as a template for the production of all the *sd* deletion constructs in this study unless otherwise specified. The ORF of *sd* in pET16b (obtained from Andrew Simmonds) was used as a template to generate full length *sd* for cloning into pUC19. The bases adenine (A) and thymine (T) which constitute part of the ATG start codon were missing from the *sd* ORF in pET16b. Therefore, primers were designed to add AT back into the full length *sd* ORF along with a *Bam*HI site for cloning into pUC19.

Primer Sd-full-1 used to amplify the 5' end of *sd* and to add AT back into the ORF is as follows (5' ⟶ 3'):

GGGATCCATGAAAAACATCACCAGCT (melting temperature-T_m is 80^{0}C)

Primer Sd-full-2 used to amplify the 3' end of *sd* is as follows (5' ⟶ 3'):

CGGCGGATCCATGCAGCTTTTGCTAT (T_m is 80^{0}C)

Note: The red sequences in the primers indicate the *Bam*HI sites, the blue colour represents the A and T missing being added back to the *sd* ORF, and orange bases outline the stop codon.

The PCR protocol used to generate the full length *sd* ORF includes a hot start at 94°C for one minute, followed by 35 cycles at 94°C for 30 seconds, 69°C for 30 seconds, 68°C for one minute and 30 seconds, a final cycle at 72°C for five minutes, and held at 4°C in a final reaction volume of 20µL. Unless otherwise indicated, all the PCR reactions used to generate the various *sd* deletions were in a final volume of 20µL. After the PCR reaction, the amplified fragments were electrophoresed to verify the size of the product. The appropriate bands were then isolated from the agarose gel and purified with the Qiaquick Gel Extraction Kit. The purified products were ligated into pUC19 using *Bam*HI. Before the ligation reaction, *Bam*HI digested pUC19 was treated with shrimp alkaline phosphatase (SAP) from United States Biochemical (USB) to prevent re-circularization (SAP protocol available at end of chapter). Subsequently, 25% of the ligation mix was used to transform cDH5α. Plasmid DNA was isolated from bacterial colonies and digested with *Pst*I to determine the presence and orientation of the insert. The correct construct was then sequenced to ensure the *sd* ORF was correct with nucleotides A and T added into it. The pUC19 vector was chosen because it is smaller and is more successful when constructing internal deletions (described below).

2.5 Construction of full length *sd*, Δ1-87, Δ345-440, and Δ1-200 into pUAST without a FLAG epitope

For construction of full length *sd*, the *sd* ORF was removed from pUC19 with a *Bam*HI digestion and ligated into *Bgl*II digested pUAST. Before the ligation step, the *Bgl*II digested vector was treated with shrimp alkaline phosphatase (SAP). For construction of constructs Δ1-87, Δ345-440, and Δ1-200 the PCR cycle used is shown in Table 2.3. See Table 2.2 for the respective primers used to generate these deletions. These deletions were cloned directionally into pUAST using *Bam*HI and *Xba*I.

2.6 Construction of *sd* internal deletions Δ88-123, Δ124-159, Δ88-159blunt/not blunt, Δ 160-219 blunt/not blunt, Δ220-281, Δ282-344, and Δ220-344 into pUAST without a FLAG epitope :

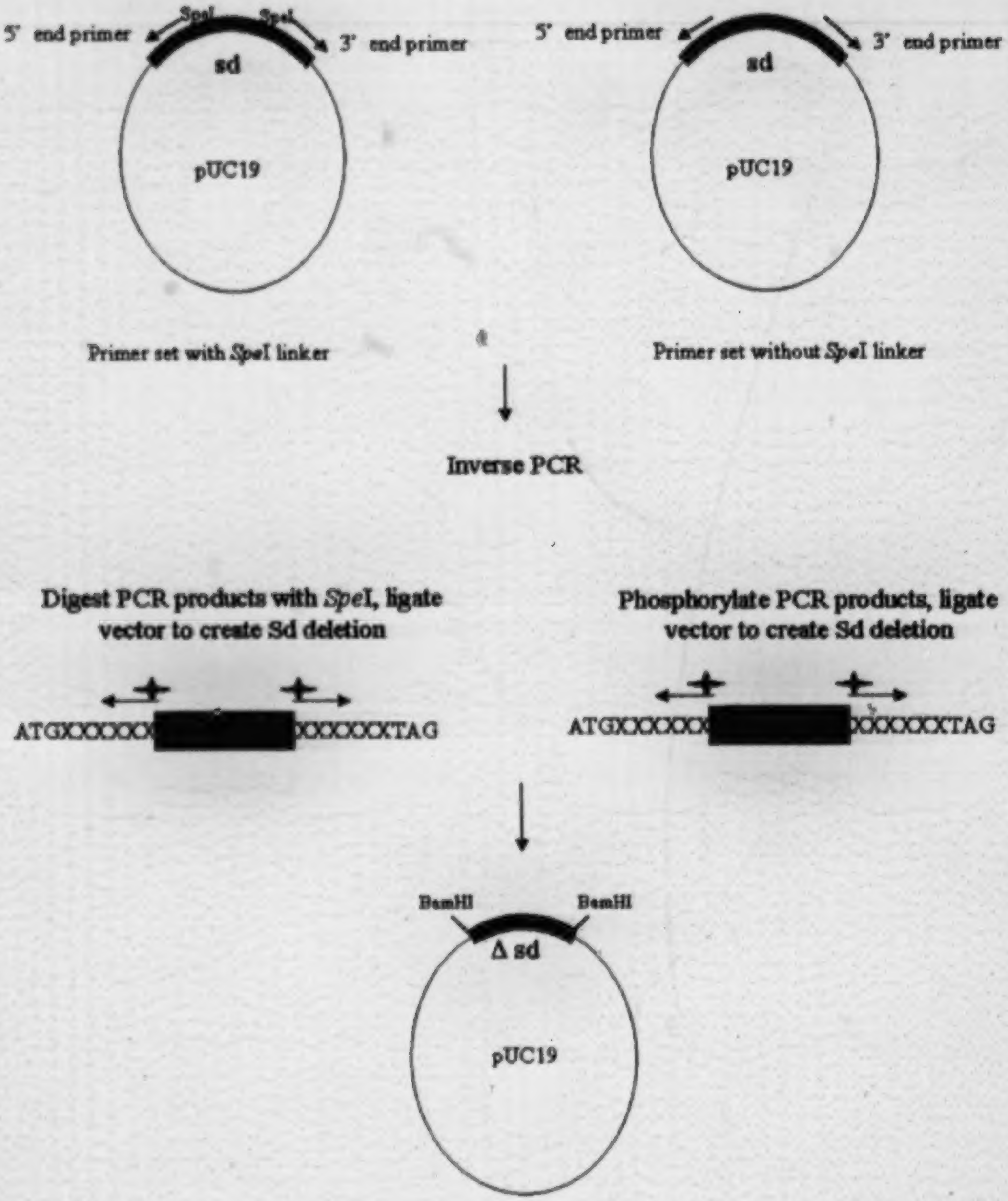
Specific primer combinations chosen to generate these deletion constructs are shown in Table 2.2. See Table 2.3 for the PCR cycle used. After the amplified PCR fragments had been isolated and purified, they were digested with *Spe*I. The digested fragments were then subjected to gel electrophoresis and the appropriate bands were isolated and purified via the Qiaquick Gel Extraction kit. Following this step, the DNA fragments were re-circularized by a ligation reaction to create the desired *sd* deletion with the ORF maintained in the pUC19 plasmid. *Bam*HI digestion was then performed to release the deleted *sd* inserts from pUC19, for subsequent ligation into *Bgl*II digested pUAST. The pUAST vector was also treated with SAP to prevent vector re-circularization before ligation with the *sd* inserts. Figure 2.4 presents a summary of this process.

Table 2.3 PCR cycles used to generate various *sd* deletion constructs

sd deletions	PCR cycles
of Δ1-87, Δ345-440, and Δ1-200	A hot start at 94^{0}C for one minute, followed by 35 cycles of 94^{0}C for 30 seconds, 69^{0}C for 30 seconds, 68^{0}C for one minute and 30 seconds, a final cycle at 72^{0}C for five minutes, and held at 4^{0}C
Δ88-123, Δ124-159, Δ88-159 not blunt, Δ 160-219 not blunt, Δ220-281, Δ282-344, Δ220-344, Δ168-219,168-219R, 88-167+345-440, Δ365-440, 88-159, 88-167, Δ434-440, Δ 416-440, Δ 391-440, 137-219, 88-219,137-344, 137-219+345-440, and 88-167+220-344	A hot start at 94^{0}C for 2 minutes, followed by 35 cycles of 94^{0}C for 30 seconds, 68^{0}C for one minute, 73^{0}C for four minutes, a final cycle at 72^{0}C for five minutes, and held at 4^{0}C
Δ88-159 blunt, and Δ 160-219 blunt	A hot start at 94^{0}C for 2 minutes, followed by 35 cycles of 94^{0}C for 30 seconds, 65^{0}C for one minute, 73^{0}C for four minutes, a final cycle at 72^{0}C for five minutes, and held at 4^{0}C
Δ345-415 and Δ416-433	A hot start at 94^{0}C for 2 minutes, followed by 35 cycles of 94^{0}C for 30 seconds, 55^{0}C for 30 seconds, 68^{0}C for five minutes, a final cycle at 72^{0}C for five minutes, and held at 4^{0}C

For optimal yield of PCR products, various PCR cycles were used to generate different *sd* deletion fragments. The constructs in red are made by inverse PCR and constructs in black are produced by PCR.

Figure 2.4: A schematic diagram that illustrates how internal *sd* deletion constructs were generated via inverse PCR. The left of the diagram represents internal constructs made with primers containing the *Spe*I linker and the right of the diagram represents internal constructs made with primers without the *Spe*I linker. The shaded sequences represent regions that would be deleted in a particular reaction. The red stars represent 5' phosphates generated by restriction digestion and the green stars illustrate 5' phosphates generated by T4 polymerase kinase reactions. The final result of these processes was to create internal *sd* deletions with the ORF maintained in the pUC19 vector.


5' end primer
SpeI
SpeI
3' end primer
sd
pUC19
Primer set with SpeI linker
5' end primer
3' end primer
sd
pUC19
Primer set without SpeI linker
Inverse PCR
Digest PCR products with SpeI, ligate vector to create Sd deletion
ATGXXXXXX
XXXXXXTAG
Phosphorylate PCR products, ligate vector to create Sd deletion
ATGXXXXXX
XXXXXXTAG
BamHI
BamHI
Δ sd
pUC19

Because the *Spe*I site engineered into the primers encodes two extra amino acids (serine and threonine) at the junction of each deletion, blunt end versions of Δ88-159 and Δ160-219 were also generated. This is to ensure that the additions of threonine and serine at the internal deletion junction had no effect on the wing rescue assay. A Δ160-219 construct made without the *Spe*I sequence will be referred to as Δ160-219 blunt (Δ160-219b), whereas a Δ160-219 construct made with the *Spe*I sequence will be designated as Δ160-219 not blunt (Δ160-219nb). This nomenclature system was also utilized for the Δ88-159 construct.

The primers used to generate blunt versions of Δ88-159 and Δ160-219 omitted the *Spe*I linker (Table 2.2). These two deletions were made in the exact manner as the rest of the internal deletion constructs with two exceptions. First, the PCR protocol used was different (Table 2.3) to increase the yield of the PCR product. Second, the step to digest the PCR product with *Spe*I was omitted. Rather, the purified PCR fragments were phosphorylated with T4 polymerase kinase (details of this reaction provided at the end of chapter) before it was re-circularized. This step was necessary to add a 5' phosphate to the linear fragments so re-circularization could occur (Figure 2.4). This step was not required when a *Spe*I linker was present since the *Spe*I digestion reaction resulted in a 5'phosphate overhang for the ligation reaction to occur.

2.7 Constructions of *sd* Δ168-219, 168-219R, Δ434-440, Δ416-440, Δ391-440, Δ365-440, 88-159, and 88-167 with 1xFLAG epitope into pUAST.

For optimal yield of PCR products, various PCR conditions were used to generate the above *sd* deletion fragments. See Table 2.3 for the different PCR cycles. The purified PCR fragments for constructs Δ168-219 and Δ168-219R (residues 168 to 219 of Sd replaced with a 156bp pBSII(SK+) fragment, see Figure 2.5 for replacement process) were subloned into pBSII(SK+)-1xFLAG plasmid with *Bam*HI. For the remainder of the constructs listed above, the purified PCR products were cloned directionally into pBSII(SK+)-1xFLAG plasmid with *Bam*HI and *Kpn*I. Following the successful cloning of all these *sd* deletions into the pBSII(SK+)-1xFLAG vector, the *sd* deletions along with the FLAG epitope were directionally cloned into pUAST with *Kpn*I and *Sst*II.

2.8 Construction of *sd* 137-219, 88-219, 137-344, Δ345-415, Δ416-433, 137-219+345-440, 88-167+220-344, and 88-167+345-440 with 2xFLAG epitope in pUAST

Various PCR conditions were used to generate the above *sd* deletion fragments for reasons stated previously. Table 2.3 shows the different PCR cycles. Not all the PCR template used was *sd*pUC19 for this group of constructs, see Table 2.2 for the respective template used for each of these constructs.

For constructs Δ345-415 and Δ416-433 which were made without the *Spe*I linker, the purified PCR products were subloned into pBSII(SK+)-1xFLAG plasmid with *Bam*HI. For the remainder of the deletion constructs, the purified PCR products were subcloned into pBSII(SK+)-1xFLAG plasmid with *Bam*HI and *Kpn*I. Subsequently, the

Figure 2.5: A diagrammatic representation of the construction of 168-219R with 1xFLAG in pUAST. After the inverse PCR reaction to generate *sd* Δ168-219 in pUC19, the PCR product was digested with *Spe*I and treated with SAP to prevent re-circularization. Notice the ligation step to re-circularize the PCR product was omitted and instead the linear fragment was ligated with a 156 base pair (bp) *Spe*I digested pBSII (SK+) fragment generated from PCR. The PCR protocol is as follows: A hot start at 94^{0}C for three minutes, 94^{0}C for 45 seconds, 65^{0}C for one minute, 72^{0}C for one minute (35 cycles), 72^{0}C for ten minutes, and held at 4^{0}C. The template for the reaction was pBSII (SK+).

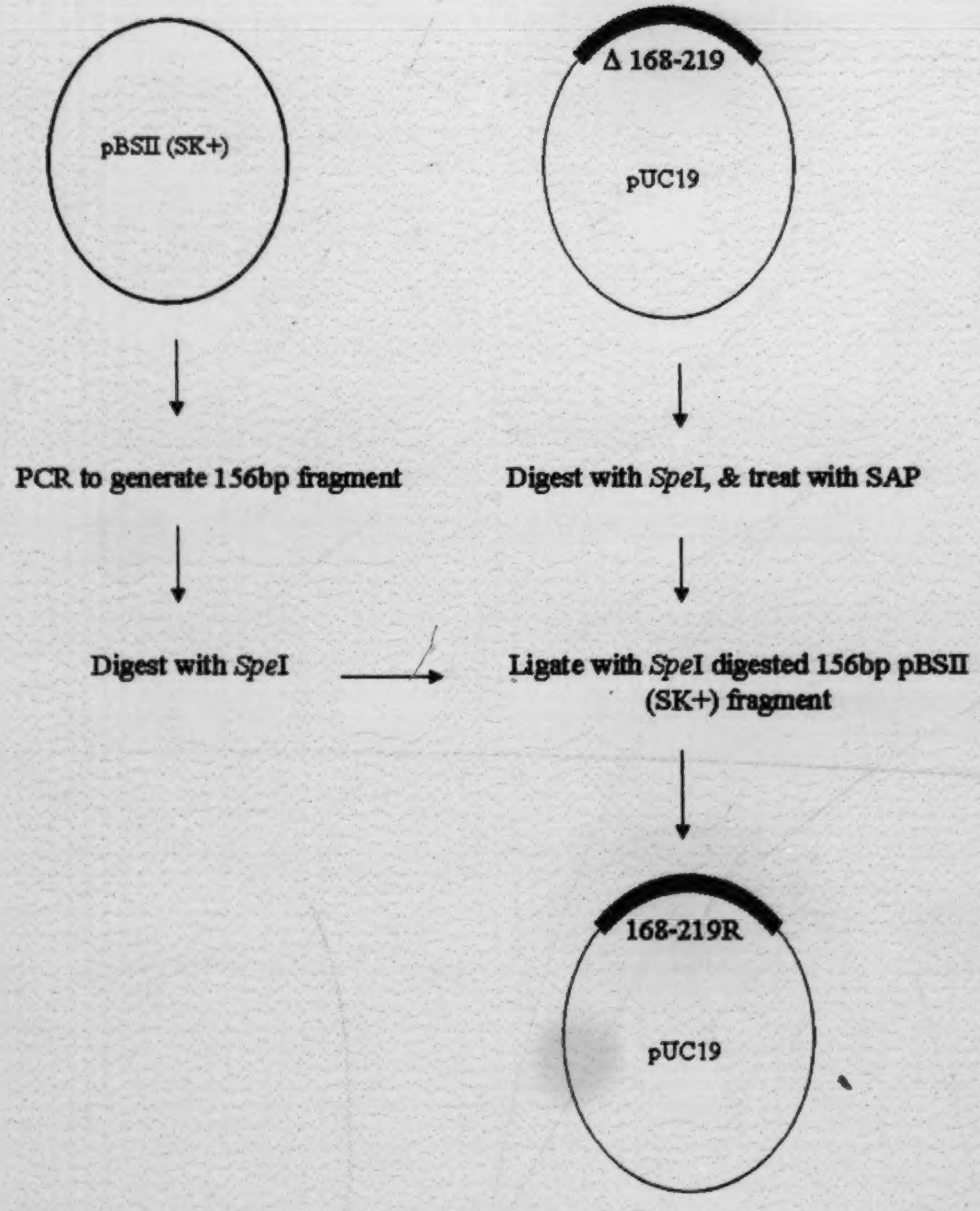
The 5'end primer used to generate the 156bp fragment is below:

$^{5'}$TCTACTAGTATAACGCAGGAAAGAACA$^{3'}$

The 3'end primer is as follows:

$^{5'}$TTTACTAGTGGTTTCGCCACCTCTG$^{3'}$

The purple color indicates the *Spe*I sites in the primers. The 156bp fragment was analyzed for the absence of stop codons and putative phosphorylation sites. Once the 156bp fragment was ligated into *sd* Δ168-219pUC19, it was cloned into pUAST in a manner similar to how *sd* Δ168-219 was ligated into pUAST. The red block represents the replacement of the *sd* linker with the 156bp fragment.


pBSII (SK+)
Δ 168-219
pUC19
PCR to generate 156bp fragment
Digest with *Spe*I, & treat with SAP
Digest with *Spe*I
Ligate with *Spe*I digested 156bp pBSII (SK+) fragment
168-219R
pUC19

sd deletions along with the FLAG epitope were directionally cloned into pUAST with *Kpn*I and *Xba*I. Construct 88-219 was digested with *Eco*RV and *Pst*I whereas 137-219, 137-344, 137-219+345-440, 88-167+220-344, and 88-167+345-440 were digested with *Eco*RV and *Pvu*II to test for the presence of the *sd* insert in pUAST.

2.9 Micro-injections (modified from MacKay, 2001):

All *sd* constructs were micro-injected into *yw* embryos, along with the helper plasmid Δ2-3. Fly population cages were assembled one to two days prior to injection to allow for sufficient egg laying. Eggs were collected on agar plates streaked with a small amount of yeast (baking yeast, water, and red wine vinegar). Microscope slides were then prepared to assemble the eggs for injections. Glycerol (80%) was used as adhesive to hold in place a cover slip at the center of the slide. Injection glue (double sided Scotch tape and heptane) was added in a strip along the two edges of the cover slip. The needles for injecting embryos were pulled with the Sutter instrument Co. Model P-87 Flaming/Brown Micropipette Puller using 50ul disposable pipettes.

For injections, fly embryos between 30 to 45 minutes old were collected, and dechorionated with a 50:50 bleach: water mixture for approximately 45 seconds with continuous agitation. These embryos were then washed thoroughly with dH_20 and lined up along the two edges of a piece of square agar the approximate size of the cover slip, with the posterior ends of the embryos facing outward. About 20 embryos were lined up per side, and were then transferred to the pre-glued cover slip. Afterwards, the embryos were dessicated in Drierite (8 mesh desiccant) for seven minutes and 45 seconds. Once dessicated, the embryos were covered with oil (Halocarbon 200 oil) for injections.

Approximately, 0.05ng to 0.25ng of DNA was injected into the posterior of each embryo (pre-blastoderm stage) and then covered with the oil again. Embryos were kept at 18^0C for two to three days and then the larvae were transferred to food (formula 4-24 instant *Drosophila* media from Carolina Biological Supply Company)

2.10 Precipitation of DNA for micro-injections:

Approximately 25μg of ethanol precipitated plasmid DNA was used for preparation for all micro-injections. In addition, 5μg of Δ2-3 helper vector was added to the plasmid DNA, and the reaction volume brought to 30 μL with milliQ H_2O. Next, 200 mM NaCl and 2.5x volume of 95% ethanol were added to the sample, followed by incubation on ice for at least 30 minutes. The sample was then centrifuged at 13200 rpm for five minutes, washed with 500uL of 70% ethanol, and air dried for five minutes. This was followed by re-suspension of the DNA pellet in 20 μL of milliQ H_20.

2.11 Ethanol precipitation protocol:

A 10% volume of 3M NaAc and 2x volume of 95% ethanol were added to the DNA to be purified. The sample was then incubated at -20^0C for at least 30 minutes, followed by centrifugation at 13200 rpm for ten minutes. The DNA pellet was then washed again with 1x volume of 75% ethanol, and allowed to air dry. A suitable amount of milliQ H_20 was used to dissolve the DNA pellet.

2.12 RT-PCR:

Transgenic flies harboring the following constructs were tested for the presence of the relevant *sd* transcript since these constructs were not tagged with a FLAG epitope and a Sd antibody is not available. These constructs included full length *sd*, Δ1-87, Δ1-200, Δ88-123, Δ124-159, Δ 88-159blunt/not blunt, Δ 160-219blunt/not blunt, Δ 220-281, Δ 220-344, Δ 282-344, and Δ 345-440. F_1 third instar larvae from the wing rescue experiments were collected. RNA was isolated using Trizol (Invitrogen) followed by DNaseI (Amplification Grade Invitrogen) treatment (described below). RNA samples were then used to generate cDNA using a Chr14 primer (Table 2.4) with or without Reverse Transcriptase Superscript (II) (Invitrogen). After the synthesis of the cDNA, PCR was performed with primers Chr 15 and Chr16 (Table 2.4) to test for quality and possible DNA contamination of the RNA samples. Primer Chr14, Chr 15, and Chr 16 annealed to ribosomal protein 49 (RP49) transcripts. The PCR cycle used is as follows: A hot start at 94^0C for one minute and thirty seconds, followed by 25 cycles of 94^0C for 30 seconds, 61^0C for 45 seconds, 72^0C for one minute and thirty seconds, a final cycle at 72^0C for five minutes, and held at 4^0C in a reaction volume of 20μL. After these tests, cDNA was synthesized from these RNA samples using primer Sd full-2 (Table 2.5) which annealed to the various *sd* transgenes being tested. However for construct Δ345-440, primer 3'Δ96 was used to synthesize the cDNA. The cDNA then served as templates for the following PCR reaction: 94^0C for one minute and 30 seconds (hot start), 94^0C for 30 seconds, 58^0C for one minute, 72^0C for five minutes (35 cycles), 72^0C for five minutes and held at 4^0C in a reaction volume of 20μL. Primers named nested Sd

Table 2.4 RT-PCR primers for the RP49 transcript and corresponding T_m values

Primer name	Primer sequence 5' → 3'	T_m (^0C)
Chr14	TCTTCTTGAGACGCAGGCGA	62
Chr15	AGCATACAGGCCCAAGATCG	62
Chr16	AGTAAACGCCGGTTCTGCAT	60

Table 2.5 RT-PCR primers used to verify the *sd* transgenes and corresponding T_m values

Primer name	Primer sequence 5' → 3'	T_m (^0C)
Sd full-2	CGGCGGATCCATGCAGCTTTTGCTAT	80
3'Δ 96	AATCTAGACTAGCCAAAGGAGCAAACGA	80
Nested Sd full-2	CATATAGCGTTCCGGTAGGTTCTT	74
UAS 5'ALT	ACCAGCAACCAAGTAAATCAACTGCA	74

Primers Sd full-2, 3'Δ96, and nested Sd full-2 anneal to the *sd* transgenes whereas UAS 5' ALT anneals to sequences upstream of the multiple cloning site in pUAST.

full-2 and UAS 5'ALT (Table 2.5) were used to amplify the different *sd* transgenes. However, for construct Δ345-440, primers 3'Δ96 and UAS 5'ALT were used instead.

2.13 RNA isolation for RT-PCR:

Between three to five third instar larvae were homogenized in 1mL of Trizol reagent (Invitrogen) following the manufacturer's protocol.

2.14 DNaseI treatment and cDNA synthesis:

Isolated RNA was treated with DNase I (Invitrogen) in a 10μL reaction volume for 14 minutes at room temperature. The DNase I was inactivated by addition of 1μL of 25mM EDTA and heat treatment at 65^0C for ten minutes. Spectrophotometer readings were recorded to measure purity and concentration of the RNA samples. Between 500ng to 600ng of RNA, along with 1μL of 10mM dNTP mix, and 2 pmole of the appropriate primer were adjusted to a volume of 12μL with DEPC H_2O. The sample was then incubated at 70^0C for five minutes, cooled on ice for five minutes, followed by addition of 4μL 5x first strand buffer, and 2μL of 0.1M DTT mix (both reagents provided by Invitrogen along with SuperScript II RT). The sample was then incubated at 42^0C for two minutes, followed by addition of 1μL of SuperScript II RT (Invitrogen), and incubated at 42^0C for 50 minutes. The reaction was inactivated at 85^0C for 15 minutes, and incubated at -70^0C for at least 30 minutes. The sample could then be used as template for PCR reactions.

2.15 Western blot analysis

Several of the FLAG tagged *sd* constructs were generated with a FLAG epitope and were found to be translated in transgenic flies. These included constructs Δ168-219, Δ168-219R, Δ416-433, Δ345-415, Δ434-440, Δ416-440, Δ391-440, Δ365-440, 137-344, 88-167+220-344, 88-167+345-440, 137-219+345-440, 88-219, 137-219, 88-159, and 88-167. To express these deletion constructs, a *hs*Gal4 driver which can express the transgenes in all tissues of the fly was used. Between three to five third instar larvae per construct were subjected to the following heat shock cycle; 37^0C for 30 minutes, followed by a resting period at room temperature for 30 minutes, heat shock at 37^0C for 30 minutes, and recovery at room temperature for 50 minutes. The larvae were then homogenized in 1.5X SDS reducing buffer (3.8mL of deionized H_2O, 0.8mL glycerol, 1.6mL 10% SDS, 0.4mL 2-mercaptoethanol, 0.4mL 1% bromophenol blue, 1.0 mL of 0.5M Tris-HCL pH6.8 to make a final volume of 8.0mL stock solution). This was followed by heat treatment at 95^0C for ten minutes, centrifugation at 13000rpm for 4 minutes, and 50% of the supernatant was heated at 98^0C for five minutes before loading onto SDS polyacrylamide gels. The products from constructs Δ168-219R, Δ168-219, Δ416-433, Δ345-415, Δ434-440, Δ416-440, Δ391-440, Δ365-440 were loaded onto 10% SDS polyacrylamide gels, while products from constructs 88-167+220-344, 137-344, 88-167+345-440, 137-219+345-440 were loaded onto 12% SDS polyacrylamide gels, and products from constructs 88-219, 137-219, 88-159, and 88-167 were loaded onto 15% SDS polyacrylamide gels. Different gel concentrations were used to suit the different expected sizes of the Sd deletion proteins. The proteins were then transferred onto nitrocellulose membranes, washed briefly with 1xPBS (10xPBS=18.6mM NaH_2PO_4, 84.1mM Na_2HPO_4, 1.75M NaCl, pH7.4) and blocked with PBT+0.1% skim milk (1xPBS, 10% Tween 20, and 0.1% skim milk powder) for 90 minutes at room temperature. The membranes were then incubated with a 1/500 dilution of anti-FLAG mouse primary antibody (Sigma) over night at 4^0C. The membrane was then washed four times with PBT+0.1% skim milk (15 minutes per wash) at room temperature. The anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (1/50000) (Jackson ImmunoResearch) was then incubated with the membrane for 90 minutes at room temperature. Afterwards, the membranes were washed four times with PBT+0.1% skim milk (15 minutes per wash), and one final wash with PBT (1xPBS+10% Tween 20) for ten minutes at room temperature. The FLAG signal was detected with Super Signal West Pico Chemiluminescent Substrate (Pierce).

2.16 Wing Rescue assays:

The UAS-Gal4 system (Brand and Perrimon, 1993) was utilized to express different *sd* deletion constructs (Table 3.1a to c) in *sd*^{ETX4} and *sd*^{58d} flies using the *vg*Gal4 driver. This wing specific driver contains the *vg*BE (Hoffman-personal communication) and thus expresses Gal4 and the *sd* transgenes along the D/V boundary of the third instar wing disc. This driver was chosen because its expression pattern recapitulates *sd* expression in the third instar wing disc.

Virgin females containing a *vg*Gal4 driver in the two tester *sd* mutant backgrounds were crossed to males carrying the various UAS-*sd* constructs (Figure 2.6). Only F_1 male flies were assayed for wing phenotypes since *sd* is located on the X chromosome and all the male progeny would be hemizygous for the *sd* tester mutations.

Figure 2.6: Schematic diagram of the cross in the rescue assay. *sd* is on the X chromosome. Therefore, when males (♂) carrying the *sd* deletion construct were crossed with sd^{ETX4} or sd^{58d} virgin females (♀) that also carried a vgGal4 construct, only F1 ♂'s were scored for the rescue outcome since they would be hemizyous for sd^{ETX4} or sd^{58d}. The wings of these F1 ♂'s were then compared to the wings of the mutant males carrying only the respective *sd* allele.

$$\begin{tikzcd}
\dfrac{y\ w\ sd^{+}}{\rightharpoondown}\ ;\ \dfrac{\mathrm{UAS}\ \Delta\ sd}{\mathrm{UAS}\ \Delta\ sd}\quad \mathrm{x}\quad \dfrac{y\ w\ sd^{ETX4/58d}}{y\ w\ sd^{ETX4/58d}}\ ;\ \dfrac{\mathrm{vgGal4}}{\mathrm{vgGal4}} \arrow[d] \\
\dfrac{y\ w\ sd^{ETX4/58d}}{\rightharpoondown}\ ;\ \dfrac{\mathrm{UAS}\ \Delta\ sd}{\mathrm{vgGal4}}
\end{tikzcd}$$

At least 100 wings were observed for each *sd* construct. Individual wings were mounted in Gary's Magic Mountant (Ashburner, 1989) and allowed to dry over night before being photographed with a Leica DMRXA light microscope.

2.17 Ectopic expression assay:

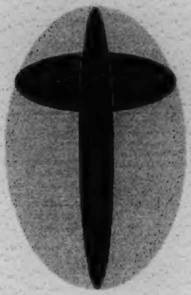
Various *sd* constructs (Table 4.1a to b) were ectopically expressed using the *ptc*Gal4 driver. The *ptc*Gal4 driver was used to express a *lac*Z reporter along the A/P boundary of the third instar wing disc (Figure 4.1c) which is different from the endogenous expression of Sd found in the wing pouch (Figure 4.1d). This driver also expresses the deletion constructs very early in development, beginning in the cellularizing blastoderm with a uniform distribution of transcription activation in the embryo (Nakano et al., 1989). The transcription pattern becomes restricted to the anterior boundary and near the posterior end of each segment during the onset of germ-band shortening, and this pattern persists until the end of embryogenesis (Nakano et al., 1989).

Female virgins harboring a *ptc*Gal4 driver in a sd^{ETX4} background were mated to various transgenic males carrying the various UAS-*sd* deletions (Figure 2.7) to assay for ectopic expression of endogenous sd^{ETX4} along the A/P boundary in third instar wing discs. The F_1 larvae were stained with X-gal to assess the ability of different regions of *sd* to induce ectopic endogenous sd^{ETX4} expression. This assay utilized the P[ry^{+}*lac*Z] element inserted in the first intron of sd^{ETX4} flies as the reporter. If ectopic expression of any of the *sd* deletion construct was able to induce ectopic expression of the endogenous sd^{ETX4} allele, there will be activation of *lac*Z along the A/P boundary of the wing disc where endogenous *sd* expression is not normally found.

Figure 2.7: A schematic diagram of the cross used in the *sd* ectopic expression assay. Virgin sd^{ETX4} females with the *ptc*Gal4 driver were crossed to males carrying each Sd deletion construct. The F1's were screened for ectopic expression of endogenous sd^{ETX4} along the A/P boundary indicated by red (i.e. the normal region of *ptc* expression in the wing disc). The green symbolizes the normal endogenous *sd* expression. The P [ry^{+}*lacZ*] element in the first intron of the sd^{ETX4} allele serves as the reporter for this assay.

$$\frac{sd^{ETX4}}{sd^{ETX4}};\frac{ptc\mathrm{Gal4}}{ptc\mathrm{Gal4}} \quad \mathrm{x} \quad \frac{y\;w\;sd^{+}}{\rightharpoondown};\frac{\mathrm{UAS}\;\Delta\;sd}{\mathrm{UAS}\;\Delta\;sd}$$

$$\downarrow$$



F1's

$$\frac{sd^{ETX4}}{y\;w\;sd^{+}};\frac{\mathrm{UAS}\;\Delta sd}{ptc\mathrm{Gal4}}$$

or

$$\frac{sd^{ETX4}}{\rightharpoondown};\frac{\mathrm{UAS}\;\Delta sd}{ptc\mathrm{Gal4}}$$

2.18 X-Gal staining (modified from Genetics 375 lab manual from University of Alberta, 1999):

Six to eight third instar larvae were collected and dissected by dividing the larvae into two halves and inverting the anterior portion. The anterior portions with wing discs attached were transferred to 493µL of 1xPBS on ice. Afterwards, 7.5µL of 50% gluteraldehyde was added and incubated for 20 minutes at room temperature. After the fixative step, the larval heads were washed three times (5 minutes per wash) with 1xPBT (1xPBS, 0.05% Triton X-100), and stained with 300µL of staining solution (10mM Na_2HPO_4, 150mM NaCl, 1mM $MgCl_2$, 5mM $K_3[Fe(CN)_6]$, 5mM $K_4[Fe(CN)_6]$) and 8µL of 8% X-gal at 37^0C for 60 to 120 minutes. The stain was then removed and the heads were washed two times (five minutes per wash) with 500µL PBT. The wing imaginal discs were dissected and mounted in 80% glycerol.

2.19 Over-expression assay in the compound eyes:

Sd constructs (Table 5.1a to c) were over-expressed in the compound eye with an *ey*Gal4 driver. This driver expresses Gal4 and hence drives expression of the various *sd* constructs starting at stage 15 of the embryo (Adachi et al., 2003). Staging is according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985). At this early stage, expression is restricted to the eye-antennal disc precursor cells (Adachi et al., 2003; Hauck et al., 1999). In the third instar larvae, this enhancer drives expression of the *sd* transgene in the eye imaginal disc and a stripe in the antennal disc, in the optic lobes of the brain and in spots in the ventral ganglion (Adachi et al., 2003; Hauck et al., 1999).

Virgin females of genotype *y w*; *ey*Gal4 UAS-Flp;GMR-hid FRT82B/TM2 were crossed to males carrying the various UAS-*sd* deletion constructs (Figure 2.8). F_1 flies carrying a maternally derived copy of *ey*Gal4, TM2 and a paternally derived copy of the UAS-*sd* construct were screened for the presence or absence of an eye phenotype.

2.20 Preparation of *Drosophila* for images of the compound eyes:

Whole flies were incubated in fixative (0.2% Tween 20, 2% formaldehyde, 2% gluteraldehyde) for at least three hours. The flies were then washed two times in 1xPBS (30 minutes per wash) before being photographed by a Philips XL Scanning Electron Microscope.

2.21 Restriction digests and treatment with SAP (USB):

All restriction digestions were carried out at 37^0C for two hours and thirty minutes to allow for complete digestion with the appropriate enzyme(s) and reaction buffer. To prevent vector re-circularization, SAP was added to the digestion reactions after two hours at 37^0C. The samples were then incubated at 37^0C for one hour and thirty minutes, followed by inactivation at 75^0C for twenty minutes.

Figure 2.8: Schematic diagram of the cross used in the eye over-expression assay. The F1's were screened for any eye and head phenotypes. The female parental genotype is actually *yw*; *ey*Gal4 UAS-Flp; GMR-hid FRT82B/TM2 and written as *yw*; *ey*Gal4 for simplicity. Only F1's carrying a copy of *ey*Gal4 and TM2 were screened.

$$\frac{y\,w}{\rightharpoonup}\,;\,\frac{\mathrm{UAS}\,\Delta\,sd}{\mathrm{UAS}\,\Delta\,sd}\ \times\ \frac{y\,w}{y\,w}\,;\,\frac{ey\mathrm{Gal4}}{ey\mathrm{Gal4}}$$

$$\downarrow$$

$$\text{F1's}\ \ \frac{y\,w}{\rightharpoonup}\,;\,\frac{\mathrm{UAS}\,\Delta\,sd}{ey\mathrm{Gal4}}$$

$$\frac{y\,w}{y\,w}\,;\,\frac{\mathrm{UAS}\,\Delta\,sd}{ey\mathrm{Gal4}}$$

2.22 Ligations and DNA transformations:

A total reaction volume of 20µL was used for all ligation reactions, varying the insert:vector ratio (1:1, 3:1, 5:1, 10:1) in order to optimize ligation efficiency. Incubation conditions were 4^0C over night. For transformation, 5µL or 25% of the ligation reaction was added to 100µL of competent *E. coli* (DH5α) cells. The mixture was incubated on ice for ten minutes, heat shocked at 42^0C for 90 seconds, followed by incubation on ice for two minutes. 700µL of warmed LB broth (37^0C) was then added to prepare for incubation at 37^0C for 25 minutes. Afterwards, 1%, 10%, and 90% of the transformation reaction were plated on LB-Ampicillin plates and incubated at 37^0C over night.

2.23 Sequencing reactions:

Between 0.5 to 1.0 µg of template DNA to be sequenced was added to the sequencing reaction consisting of 8 pmol of the sequencing primer, 4µL of sequencing buffer (200mM Tris pH 9.0, 5mM $MgCl_2$), 4µL of DYEnamic^{TM} ET terminator dye in a final volume adjusted to 20µL with milliQ H_20. This reaction was then subjected to the following PCR protocol: 95^0C for 20 seconds, 50^0C for 15 seconds, 60^0C for one minute cycled 30 times. After the PCR reaction, 0.2µg of glycogen, 2µL of NaAc/EDTA, and 80µL of 95% ethanol were added to precipitate the DNA. The sample was then centrifuged for 15 minutes at 13200 rpm, washed again with 300 µL of 70% ethanol, centrifuged for five minutes at 13200 rpm, and air dried for five minutes.

2.24 Phosphorylation reactions

Samples to be phosphorylated were combined with 1mM ATP, 1x forward buffer (Invitrogen), and the appropriate amount of T4 polymerase kinase (Invitrogen) in a reaction volume of 25μL. The reaction was then incubated at 37^{0}C for 15 minutes, and heat inactivated at 65^{0}C for ten minutes.

2.25 Maxi plasmid purifications:

DNA for micro-injections was prepared via the Qiagen Plasmid Maxi kit as per supplied instruction.

2.26 Mini plasmid preparations:

For other cloning and subcloning applications, the Qiaprep Spin Miniprep kit was used as per supplied instructions. Restriction digestions were then carried out to ensure the correct plasmid had been amplified.

2.27 DNA extraction from agarose gels:

After isolation of the appropriate DNA fragments from an agarose gel, the Qiaquick Gel Extraction Kit was used to purify the DNA from the agarose with the supplied instructions. After purification of the DNA, 10% of the sample was then subjected to electrophoresis to test for the efficiency of the purification process.

Chapter 3: An *in vivo* functional dissection of the role of Sd in *Drosophila* wing development

3.1 Introduction

In cells that will become wing tissue, Sd and Vg are believed to form a tetrameric complex consisting of two molecules of each protein (Halder and Carroll, 2001) that activates downstream wing differentiation target genes such as *cut*, and *spalt* (Halder et al., 1998). While the expression of *sd* is diverse, *vg* expression is limited to the wing and haltere disc (Williams et al., 1991). It is only when both Sd and Vg are present in the same cell that wing tissue is produced (Halder et al., 1998; Simmonds et al., 1998). For example, ectopic expression of *vg* in the eye results in wing tissue outgrowth (Simmonds et al., 1998).

For wing differentiation, Sd provides a DNA binding domain (Campbell et al., 1992; Halder and Carroll, 2001) while Vg provides the activating component of the selector complex (Halder and Carroll, 2001; Simmonds et al., 1998; Vaudin et al., 1999). It is also thought that when Vg is associated with Sd, Vg does not make contact with the DNA (Halder and Carroll, 2001). In addition to providing the DNA binding function for the Sd-Vg complex, Sd contains a putative nuclear localization signal (NLS) and is required to localize Vg to the nucleus (Srivastava et al., 2004). It is thought that when Vg binds Sd, this causes a conformational change in Sd so it binds only to relevant Sd-Vg target sites rather than other sites that may be occupied by Sd alone (Halder and Carroll, 2001). Sd-Vg sites are referred to as B sites whereas sites that are occupied by Sd alone are referred as A sites (Halder and Carroll, 2001). A sites are generally singlets whereas B sites are tandem doublet repeats (Halder and Carroll, 2001). It seems there are no consensus sequence motifs found in the A and B sites (Halder and Carroll, 2001). Examples of these sites are shown in Figure 3.1.

The Sd-Vg complex is dynamically regulated during development of the wing imaginal disc. During the mid second instar stage, the Sd-Vg complex is active at the D/V boundary of the wing disc for the proper development of the future wing margin (Vaudin et al., 1999). At the third instar stage, the activity of the Sd-Vg complex is down-regulated at the D/V boundary, and it is up-regulated in the wing pouch for the proper formation of the wing blade (Vaudin et al., 1999). At the end of the third instar stage and the prepupal stage, the Sd-Vg complex is thought to be involved in determination of the vein/intervein cell fate (Vaudin et al., 1999). In addition to organizing the wing fate, cells within the wing pouch may also require Sd for survival and growth (Liu et al., 2000). Finally, Sd may also be involved in regulation of apoptosis in the wing imaginal disc as ectopic expression of *sd* induces extensive apoptosis in this tissue (Liu et al., 2000).

Although considerable effort has been expended to determine how the Sd-Vg complex functions to dictate proper wing morphogenesis, it remains uncertain how these two proteins interact to carry out this function. Therefore, to try to gain a further understanding of the mechanism of this process, a functional dissection of Sd was carried out by assaying the ability of different deletions of this protein to rescue two *sd* recessive viable wing mutants, sd^{ETX4} and sd^{58d} (Figure 3.2). The sd^{ETX4} allele is hypomorphic and causes defects along the wing margin (Campbell et al., 1992), and a general taste defect (Anand et al., 1990). The genetic mutation associated with this allele is described in the Material and Methods section 2.17. The second allele, sd^{58d} is also hypomorphic and

Figure 3.1: Examples of A and B sites in a and b, respectively. The Sd-Vg complex binds B sites whereas Sd alone binds A sites. There are no consensus motifs within the A or B sites except that B sites are doublets whereas A sites are singlets. The red bases represent Sd binding sites (Butler and Ordahl, 1999). Sequences one to three from the A sites represent cis regulatory elements from the *spalt* (*sal*), *cardiac troponin T* (*cTNT*), and *α-myosin heavy-chain* (*αMHC*) genes, respectively. The first two sequences from the B sites represent regulatory elements from *cut* and the last sequence is a regulatory element from *sal* (Figure adapted from Halder and Carroll, 2001).

a. A sites

CATA	ACTTATTA	AAAA
AGAG	AGGAATGC	AACA
CACG	TGGAATGA	GCTA

b. B sites

TCAA	TGTAATTC	G	AAAAATGT	CGTC
CAGA	TAAAATTA	T	TGAAATTA	CATT
TTTC	TGGAATCC	C	ACGAATGT	CCAT

Figure 3.2: Examples of a sd^{ETX4} wing in a, and a sd^{58d} wing in b. The scale bar represents 300μm, as with all scale bars used hereafter in this chapter unless stated otherwise.

results from an inversion which causes the wings to be reduced to a small growth (Campbell et al., 1992). These two mutants were chosen for several reasons. First, the goal of the project was to study the mechanism of the Sd-Vg complex function in wing development. Therefore, a *sd* allele with a wing phenotype is necessary. Secondly, these two mutant alleles are recessive and viable, making the rescue assay easy to perform. Thirdly, the wing phenotypes associated with these two mutants can be readily identified. Finally, even slight perturbations in Sd-Vg function may easily modify these phenotypes.

3.2 Results

3.2-1 Definition of terms used to describe the results of the wing rescue assay and the controls used in this experiment

To assess which regions of Sd are required to rescue *sd*^{ETX4} and *sd*^{58d}, the wing rescue assay was performed as described in Materials and Methods (section 2.16). A cross was scored as rescue when the resulting wing phenotype is less severe than *sd*^{ETX4} or *sd*^{58d} flies upon expression of the transgene. "No rescue" can be further subdivided into two groups; no effect and dominant negative ("anti-rescue"). No effect is defined as when the wing phenotype does not change upon expression of the transgene whereas dominant negative indicates that the wing phenotype is more severe than *sd*^{ETX4} and *sd*^{58d} flies upon expression of the transgene. The degree of rescue or dominant negative phenotype was assigned an arbitrary value as shown in Figure 3.3. The numerical value changes from -1 to -7 as the malformation of the wings progressively worsens.

Table 3.1 lists all the deletion constructs and the various transgenic lines, chromosomal locations, and rescue results of the constructs. Transgenic larvae tested with RT-PCR were able to transcribe the corresponding Sd transgene except for the negative control pUAST which, as expected, did not produce any RNA transcript (Figure 3.4). The rest of the constructs tested with Western blot analysis were able to express the corresponding Sd protein close to the predicted size (Figure 3.5). The only exception was construct 88-219 (predicted to produce an approximately 15 KDa protein), which resulted in a band closer to 25 KDa. A possible explanation might be that these bands are actually multimers of the protein which could effect migration of the protein in the gel. The protein size prediction program used was ExPASy-tool. The rescue results also indicate

Figure 3.3: The scale used to categorize the extent of either a rescue or dominant negative phenotypic outcome from the rescue crosses. (a) indicates wildtype wings, -1 to -7 in panels b to h, respectively, indicate an increasing severity of defects in the wings. *sd*^{ETX4} wings would be scored as -2, and *sd*^{58d} wings would be scored as -6 according to this arbitrary scale. Thus a dominant negative effect for *sd*^{ETX4} wings would be -3 to -7, and for *sd*^{58d} wings would be -7. Panels e and f are each composed of two images.

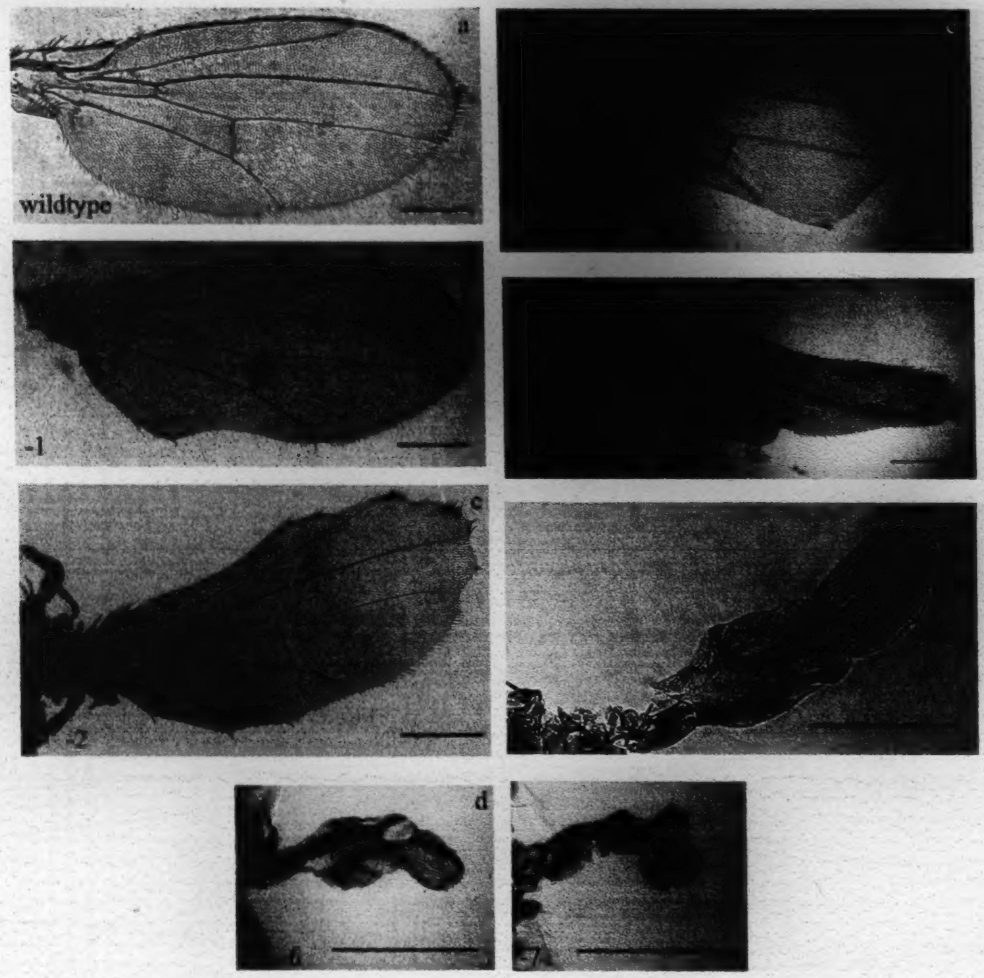

a
wildtype
c
-1
-2
c
d
6
-7

Table 3.1a Summary of the rescue results from each independent transgenic line of the deletion constructs

Constructs	Domain(s) interrupted	Line	Chromosomal Location	Rescue Results	
				sd^{ETX4}	sd^{58d}
pUAST	NA	2-6	II	No effect	No effect
		25-1	III	No effect	No effect
		27-2	III	No effect	No effect
1-440 (full)	none	18-1	II	wildtype	wildtype
		34-1	III	-7 -1	-7 -1
Δ1-87	N-terminal	7-1	III	-7	-1 to wildtype
		7-2	II	-7 -1	-7 -1
		8-3	III	-1 to wildtype -7	-1 to wildtype -7
Δ220-281	VID	4-7	II	No effect	No effect
		23-1	II	No effect	No effect
Δ282-344	VID	1-1	II	No effect	No effect
		2-6	III	No effect	No effect
Δ220-344	VID	1-4	III	No effect	No effect
		1-6	II	No effect	No effect
		2-6	II	No effect	No effect
88-159	N & C-terminal, linker, & VID	22-1	III	-4 (15%) No effect (85%)	No effect
		40-1	III	-4 (43%) No effect (57%)	No effect
88-167	N & C-terminal, linker, & VID	8-1	III	-4 (37%) No effect (63%)	No effect
		25-1	III	-4 (18%) No effect (82%)	No effect
88-167 + 345-440	N-terminal, linker, & VID	23-1	III	-4 (48%) No effect (52%)	No effect
Δ88-123	DNA binding	2-2	III	-7	-7
		2-8	II	-7	-7
		3-1	II	-7	-7
Δ124-159	DNA binding	6-2	III	-7	-7
		13-7	III	-7	-7

Table 3.1b Summary of the rescue results from each independent transgenic line of the deletion constructs continued

Construct	Domain(s) interrupted	Line	Chromosomal Locations	Rescue	
				sd^{ETX4}	sd^{58d}
Δ88-159 Not blunt	DNA binding	9-8	II	-7	-7 (75%) No effect (25%)
		9-10	III	-7	-7 (63%) No effect (37%)
Δ88-159 blunt	DNA binding	2-1	III	-7	-7 (42%) No effect (58%)
		38-1	II	-7	-7
Δ1-200	DNA binding	5-4	II	-7	-7
		10-1	II	-7	-7
		11-2	II	-7	-7
		15-1	III	-7	-7
		34-1	II	-7	-7
88-167 + 220-344	N & C-terminal, & linker	19-1	III	No effect	No effect
		28-1	III	No effect	No effect
Δ434-440	C-terminal	17-1	II	-1	-6
		21-2	II	-1 (54%) -7 (46%)	-3 to -4 (62%) -7 (38%)
Δ416-440	C-terminal	9-2	III	No effect	No effect
		11-1	III	No effect	No effect
Δ391-440	C-terminal	9-1	III	No effect	No effect
		9-2	III	No effect	No effect
		29-1	III	No effect	No effect
Δ365-440	C-terminal	8-1	III	No effect	No effect
		24-1	II	No effect	No effect
Δ345-440	C-terminal	31-2	II	No effect	No effect
		31-4	III	No effect	No effect
Δ345-415	C-terminal	15-1	III	No effect	No effect
		21-1	II	No effect	No effect
Δ416-433	putative finger motif	23-1	III	No effect	No effect
		24-1	II	No effect	No effect
		30-1	II	No effect	No effect
Δ160-219 not blunt	linker	5-1	III	-7	-7
		6-1	II	-7	-7
		12-1	II	-7	-7
		25-1	II	-7	-7
		36-1	III	-7	-7

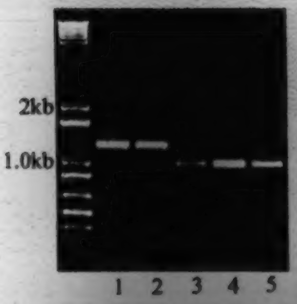
Table 3.1c Summary of the rescue results from each independent transgenic line of the deletion constructs continued

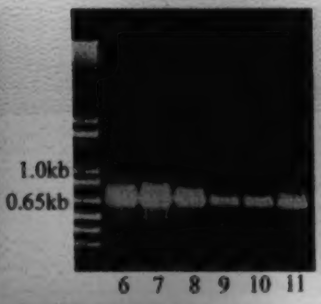
Construct	Domain(s) interrupted	Line	Chromosomal Location	Rescue	
				sd^{ETX4}	sd^{58d}
Δ160-219 blunt	linker	17-1	II	-7	-7
		24-1	II	-7	-7
		25-1	III	-7	-7
Δ168-219	linker	6-1	III	-7	-7
		9-1	III	-7	-7
		9-2	III	-7 (65%) -4 (35%)	-7 (55%) -3 to -4 (45%)
Δ168-219R	linker	39-1	III	-7	-7
		40-1	II	-7	-7 (53%) -3 to -4 (47%)
137-219	N & C-terminal, DNA binding, & VID	16-1	II	No effect	No effect
88-219	N & C-terminal, & VID	8-1	II	-4 (7%) No effect (93%)	No effect
		12-1	III	-4 (24%) No effect (76%)	No effect
		22-1	II	-4 (5%) No effect (95%)	No effect
137-344	N & C-terminal, & DNA binding	19-1	II	No effect	No effect
137-219 + 345 - 440	N-terminal, DNA binding, & VID	19-1	III	No effect	No effect
		24-1	II	No effect	No effect

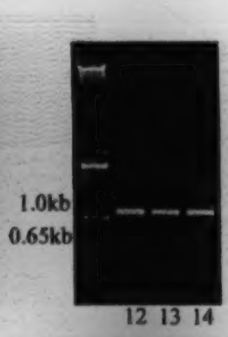
Blue designates that the rescue cross was raised at 18^{0}C, whereas red specifies that the rescue cross was done at 29^{0}C. Otherwise, all crosses were performed at room temperature. In some cases, the proportions of flies that are affected (represented as percentages) are also indicated next to the arbitrary numerical assignment. When percentages affected are not shown, 100% of the wings are affected to the same degree by expression of the respective deletion construct. Abbreviation: NA; not applicable.

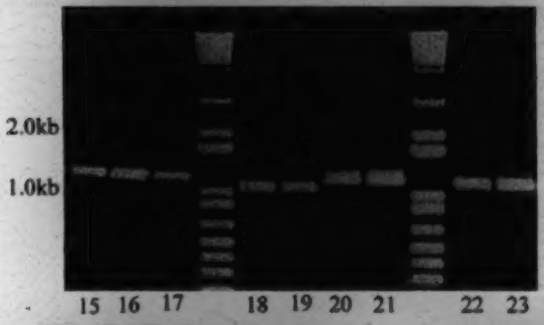
Figure 3.4: RT-PCR results to confirm that the tested constructs are transcribed in the transgenic strains.

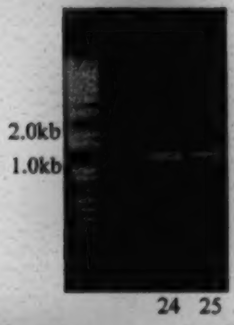
The RT-PCR results are shown for F1 third instar larvae from the rescue cross shown in the *sd*^{ETX4} background as in Figure 2.6. All RT-PCR reactions were done in an extract from a *sd*^{ETX4} background. The RT-PCR product for each construct is in agreement with the predicted size for each respective deletion. A 1kb plus ladder (Invitrogen) was used. The construct identity for individual lanes of these gel results is provided on page 85.


2kb
1.0kb
1 2 3 4 5


1.0kb
0.65kb
6 7 8 9 10 11


1.0kb
0.65kb
12 13 14


2.0kb
1.0kb
15 16 17 18 19 20 21 22 23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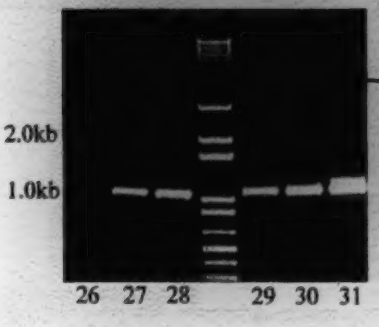
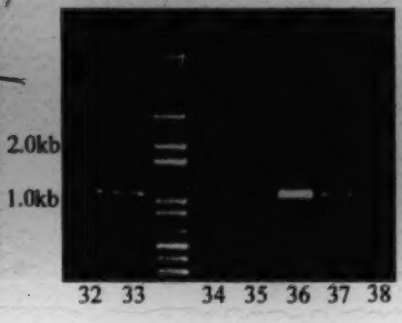
24 25


2.0kb
1.0kb
26 27 28 29 30 31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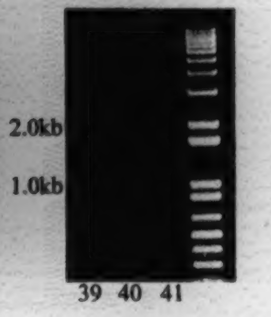
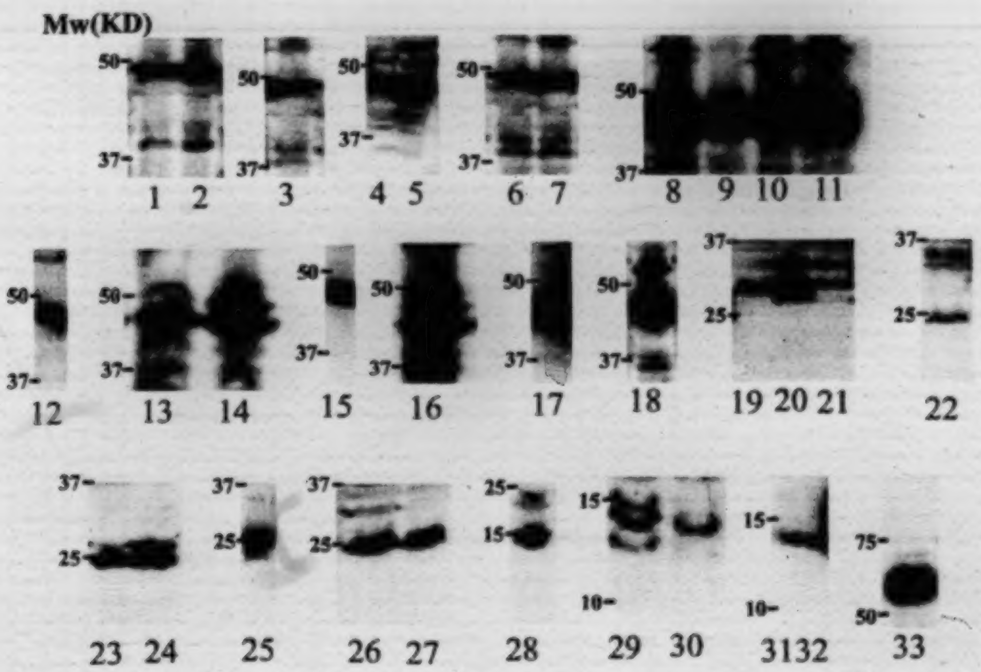
32 33 34 35 36 37 38


2.0kb
1.0kb
39 40 41

Lane number	Construct	Expected size of PCR product (base pairs)
1	1-440 #18-1	1335
2	1-440 #34-1	1335
3	Δ1-87 #7-1	1074
4	Δ1-87 #7-2	1074
5	Δ1-87 #8-3	1074
6	Δ1-200 #5-4,	735
7	Δ1-200 #10-1	735
8	Δ1-200 #11-2	735
9	Δ1-200 #15-1	735
10	Δ1-200 #34-1	735
11	Δ1-200 #35-5	735
12	Δ220-344 #1-4	960
13	Δ220-344 #1-6	960
14	Δ220-344 #2-6	960
15	Δ88-123 #2-2	1227
16	Δ88-123 #2-8	1227
17	Δ88-123 #3-1	1227
18	Δ88-159nb #9-8	1125
19	Δ88-159nb #9-10	1125
20	Δ124-159 #6-2	1224
21	Δ124-159 #13-7	1224
22	Δ220-281 #4-7	1149
23	Δ220-281 #23-1	1149
24	Δ345-440 #31-2	1032
25	Δ345-440 #31-4	1032
26	Δ88-159b #2-1	1119
27	Δ88-159b #7-1	1119
28	Δ88-159b #38-1	1119
29	Δ160-219b #17-1	1149
30	Δ160-219b #24-1	1149
31	Δ160-219b #25-1	1149
32	Δ282-344 #2-6	1146
33	Δ282-344 #1-1	1146
34	Δ160-219nb #5-1	1155
35	Δ160-219nb #6-1	1155
36	Δ160-219nb #12-1	1155
37	Δ160-219nb #25-1	1155
38	Δ160-219nb #36-1	1155
39	pUAST #2-6	0
40	pUAST #25-1	0
41	pUAST #27-2	0

Figure 3.5: Western Blot analysis to determine whether specific constructs are translated in the transgenic strains. The following deletion constructs used in the rescue assay were generated with either 1xFLAG or 2xFLAG (see Materials and Methods for details). Lane 33 represents a FLAG epitope fused in-frame to full length Vg which is known to be recognized by the Sigma FLAG antibody and serves as a positive control for the Western Blot analysis. This protein was electrophoresed in a 12% protein gel. The molecular ladder used was Precision Plus Protein Standards (Bio-Rad). The construct identity of individual lanes is provided on page 88.


Mw(KD)
50
37
1 2
3
4 5
6 7
8 9 10 11
12
13 14
15
16
17
18
19 20 21
22
25
23 24
25
26 27
28
15
29 30
10
3132
75
33

Lane number	Construct	Expected protein size (KD)
1	Δ168-219R #40-1	50
2	Δ168-219R #39-1	50
3	Δ433-440 #17-1	49
4	Δ433-440 #21-2	49
5	Δ416-433 #30-1	48
6	Δ416-433 #23-1	48
7	Δ416-433 #24-1	48
8	Δ416-440 #9-2	47
9	Δ416-440 #11-1	47
10	Δ391-440 #9-1	44
11	Δ391-440 #9-2	44
12	Δ168-219 #6-1	44
13	Δ168-219 #9-2	44
14	Δ168-219 #9-1	44
15	Δ345-415 #15-1	41
16	Δ345-415 #21-1	41
17	Δ365-440 #8-1	40
18	Δ365-440 #24-1	40
19	87-168+220-344 #28-1	24
20	87-168+220-344 #19-1	24
21	137-344 #19-1	24
22	87-168+345-440 #23-1	21
23	137-219+345-440 #24-1	21
24	137-219+345-440 #19-1	21
25	88-219 #12-1	15
26	88-219 #22-1	15
27	88-219 #8-1	15
28	137-219 #16-1	9
29	87-167 #25-1	9
30	87-159 #22-1	8
31	87-167 #8-1	9
32	87-159 #40-1	8
33	full length Vg	46

that for transgenic expressing constructs Δ88-159, and Δ160-219, the two additional amino acids encoded by the *Spe*I linker (Material and Method section 2.6) did not have an effect in the wing rescue assays (Figure 3.6 a to d, 3.7 c to f).

As a negative control for the rescue assay, the pUAST injection vector was expressed with the *vg*Gal4 driver in transgenics harboring the vector and no effect was observed in the sd^{ETX4} and sd^{58d} mutant backgrounds. In addition, full length Sd (#18-1, #34-1) transgenics were also produced to serve as positive controls for the rescue assay. Expression of full length Sd line #18-1 was able to rescue sd^{ETX4} and sd^{58d} mutant wings to resemble wildtype wings (Figure 3.8a & b). Surprisingly, expression of full length Sd line #34-1 caused a dominant negative phenotype in both of these mutant backgrounds (Figure 3.8 c & d). These wings were assigned a numerical value of -6 to -7, which indicates that the majority of the wing blades were missing (Figure 3.3) and, in addition, there was necrosis of the wing tissues. It is known that *Drosophila* wing development is very sensitive to the relative levels of Sd and Vg in the cell, and Vg is the limiting component of the Sd-Vg complex (Simmonds et al., 1998). To test whether increasing the level of Vg in the cells would alleviate the dominant negative effect caused by expression of full length Sd line #34-1, full length Vg was simultaneously expressed. Interestingly, the dominant negative effect was alleviated to between -3 and -4 wings in both mutant backgrounds when full length Vg was simultaneously expressed with full length Sd #34-1(Figure 3.8e & f). The numerical values of -3 to -4 represent wings that have progressively smaller wing blades (Figure 3.3).

Figure 3.6: Representative rescue results from specific Sd constructs that cause a dominant negative phenotype upon expression. These constructs are either interrupted in the TEA DNA binding domain or the linker region. (a) Over-expression of *sd* Δ160-219nb ($^{\#}$6-1) in sd^{ETX4} or (b) sd^{58d} wings. (c) Over-expression of *sd* Δ160-219b ($^{\#}$25-1) in sd^{ETX4} or (d) sd^{58d} mutants. Note: Δ160-219 nb indicates that the *Spe*I linker is present, and Δ160-219b indicates that the *Spe*I linker is absent. Thus, addition of the *Spe*I linker did not have any effect in the rescue results of Δ160-219. (e) Over-expression of Δ88-123 ($^{\#}$3-1) in sd^{ETX4} or (f) sd^{58d} flies. Over-expression of the injection vector pUAST had no effect in either mutant background. The control wing phenotypes for sd^{ETX4} and sd^{58d} mutants are as shown in Figure 3.2. Results for other independent lines of Δ160-219nb, Δ160-219b, and Δ88-123 are identical (data not shown).

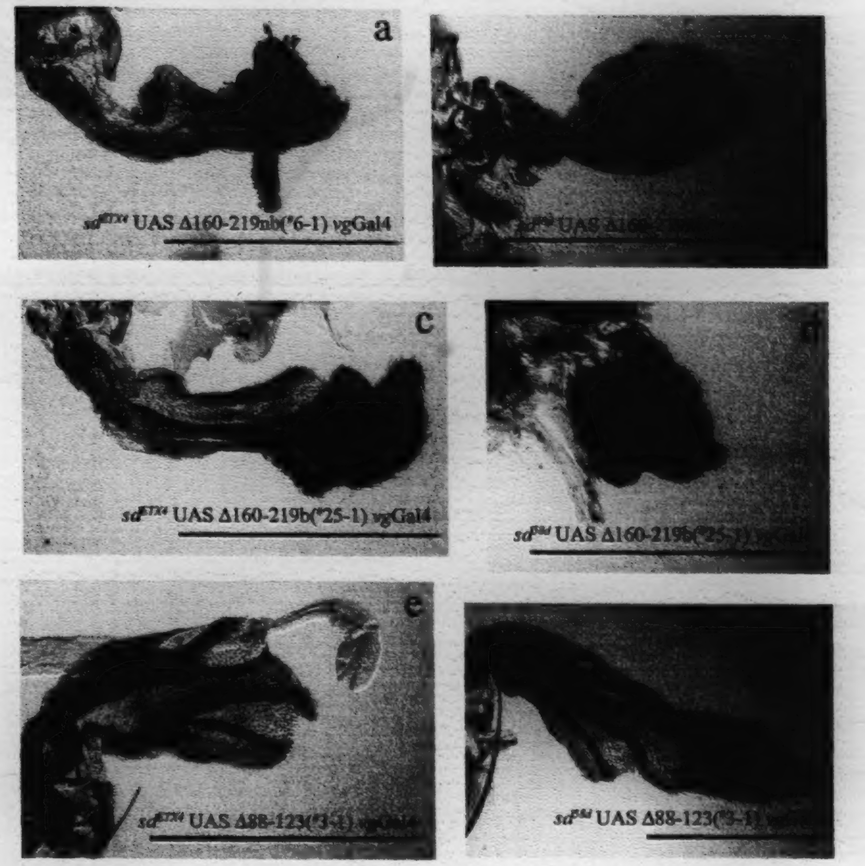

a
sd^{ETX4} UAS Δ160-219nb(*6-1) vgGal4
sd^{58d} UAS Δ160
c
sd^{ETX4} UAS Δ160-219b(*25-1) vgGal4
d
sd^{58d} UAS Δ160-219b(*25-1)
e
sd^{ETX4} UAS Δ88-123(*3-1)
sd^{58d} UAS Δ88-123(*3-1)

Figure 3.7: Expression of the following Sd constructs interrupted in the TEA DNA binding domain also cause a dominant negative phenotype in both mutant backgrounds. Furthermore, simultaneous expression of full length Vg can alleviate the dominant negative effect caused by expression of *sd* Δ1-200 (#10-1). (a) Over-expression of Δ124-159 (#6-2) in sd^{ETX4} or (b) sd^{58d} wings. (c) Over-expression of Δ88-159nb (#9-8) in sd^{ETX4} or (d) sd^{58d} mutants. (e) Over-expression of Δ88-159b (#38-1) in sd^{ETX4} or (f) sd^{58d} flies. Note: addition of the *Spe*I linker did not change the rescue outcome of Δ88-159. (g) Over-expression of *sd* Δ1-200 (#10-1) in sd^{ETX4} or (h) sd^{58d} flies. (i) Over-expression of full length *vg* along with Δ1-200 (#10-1) alleviates the dominant negative phenotype in sd^{ETX4} or (j) sd^{58d} wings. Results for other transgenic lines of Δ1-200, Δ124-159, Δ88-159nb and Δ88-159b are identical (data not shown).

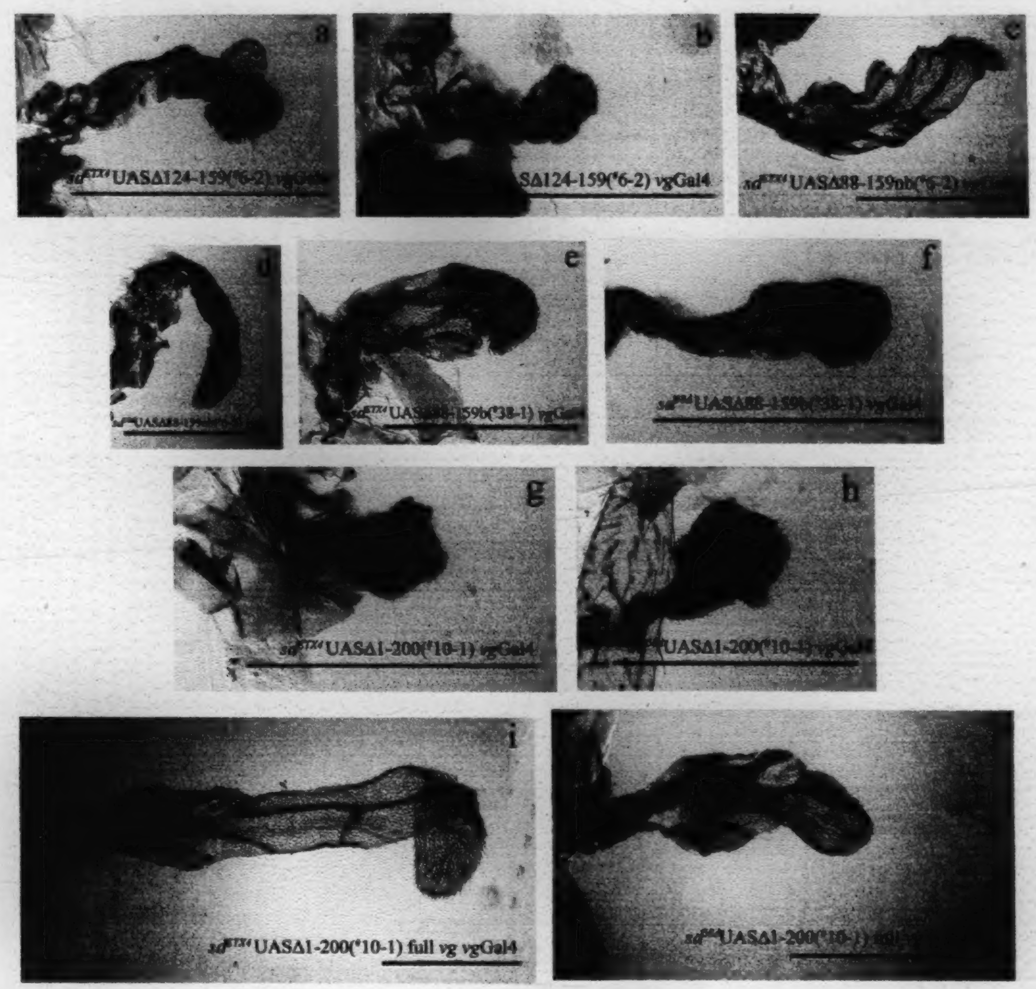
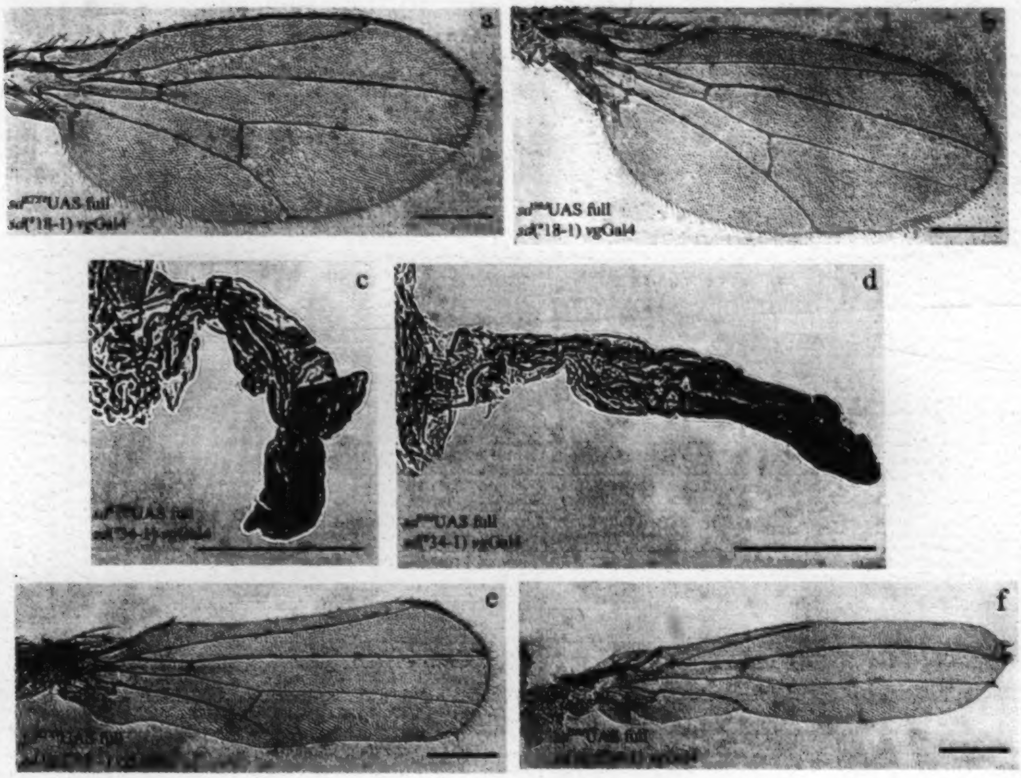

a
sd^{ETX4} UASΔ124-159(*6-2) vgGal4
b
SΔ124-159(*6-2) vgGal4
c
sd^{ETX4} UASΔ88-159nb(*6-2)
d
e
sd^{ETX4} UAS 159b(*38-1)
f
sd^{58d} UASΔ88-159b(*38-1) vgGal4
g
sd^{ETX4} UASΔ1-200(*10-1) vgGal4
h
UASΔ1-200(*10-1)
i
sd^{ETX4} UASΔ1-200(*10-1) full vg vgGal4
sd^{58d} UASΔ1-200(*10-1)

Figure 3.8: Full length Sd expression causes either rescue or a dominant negative effect in both *sd*^{ETX4} or *sd*^{58d} backgrounds depending on the respective transgenic strain. Transgenic full length Sd line #18-1 rescues *sd*^{ETX4} (a) and *sd*^{58d} (b) wings to wildtype wings. However, another independent line of full length Sd #34-1 causes a dominant negative effect (-7 wings) in both *sd*^{ETX4} (c) and *sd*^{58d} (d) mutants. The dominant negative effect was alleviated to scores of -3 to -4 wings in *sd*^{ETX4} (e) and *sd*^{58d} (f) flies when full length Vg was simultaneously expressed.

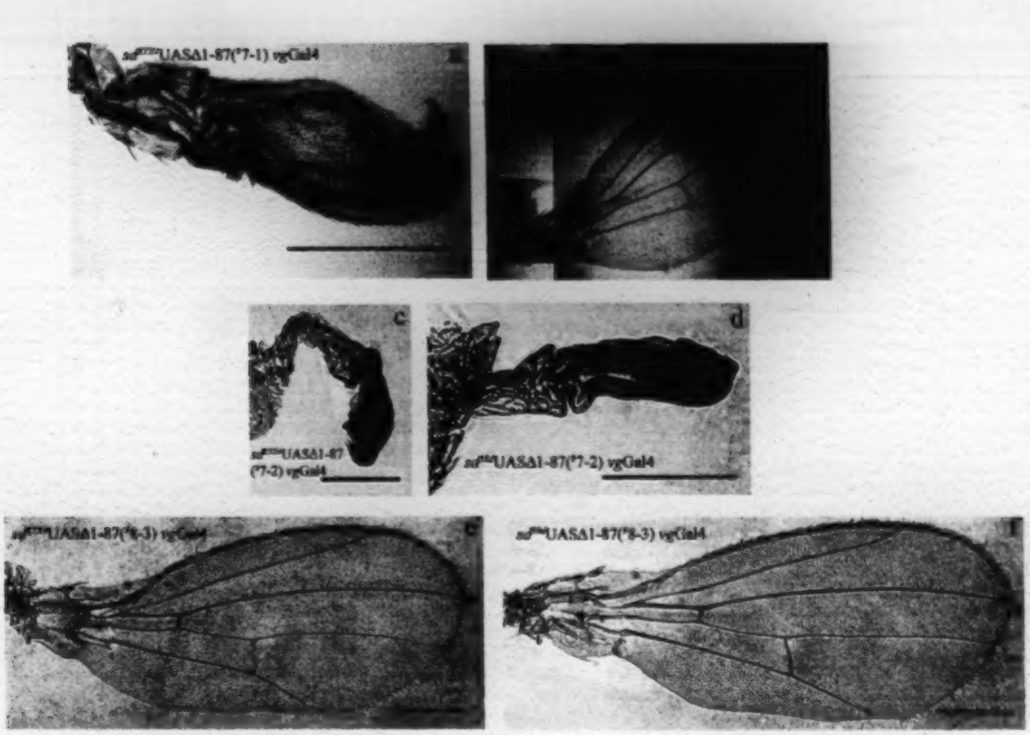

a
sd UAS full
sd(*18-1) vgGal4
b
sd UAS full
sd(*18-1) vgGal4
c
sd UAS full
d
sd UAS full
sd(*34-1) vgGal4
e
UAS full
f
UAS full

3.2-3 Rescue results for transgenic flies expressing Sd Δ1-87, and Sd interrupted in the VID

Despite the evolutionary conservation between Sd and TEF-1, the N-terminal domain is not conserved between the two proteins (Deshpande et al., 1997). To determine if the N-terminal region of Sd has any function in *Drosophila* wing development, construct Δ1-87 (#7-1, #7-2, and #8-3), which deletes the N-terminal domain of Sd up to the TEA DNA binding domain was made and tested in the rescue assay. Transgenic flies expressing this construct behaved similarly to transgenic flies expressing full length Sd. Flies expressing line #8-3 of Δ1-87 show a rescue phenotype in both mutant backgrounds (-1 to wildtype) (Figure 3.9e & & f). The numerical value of -1 indicates wings with minor and rare nicks on the wing margin (Figure 3.3). However, expression of line Δ1-87 #7-1 has a dominant negative effect in *sd*^{ETX4} mutants (-7) whereas it rescues *sd*^{58d} mutants to a phenotype of -1 to wildtype wings (Figure 3.9a & b). Expression of line #7-2 of Δ1-87 causes a dominant negative effect (-7) in both the *sd*^{ETX4} and *sd*^{58d} wings (Figure 3.9c & d).

The next region of Sd tested *in vivo* was the Vg interacting domain (VID). As mentioned in the introduction, the VID was determined by sequence alignment with the region of TEF-1 that interacted with Vgl-1 (Vaudin et al., 1999). To test whether this region of Sd is associated *in vivo* with Vg to influence wing development, part of or the entire VID of Sd was deleted. Constructs Δ220-281 (#4-7, #23-1), Δ282-344 (#1-1, #2-6), and Δ220-344 (#1-4, #1-6, #2-6) which delete the first half, the second half and the entire VID, respectively, were generated. Upon expression in transgenic flies, none of these constructs was able to affect *sd*^{ETX4} and *sd*^{58d} wings.

Figure 3.9: The N-terminal 87 amino acids of Sd are not necessary for its role in wing development since construct Δ1-87 behaves as full length Sd in the rescue assay. Line $^{\#}$7-1 of Δ1-87 has a dominant negative effect (-7 wings) in the sd^{ETX4} background (a) whereas it rescues sd^{58d} mutants to between -1 to WT wings (b). Another independent line of Δ1-87 $^{\#}$7-2 causes a dominant negative effect (-7 wings) in both sd^{ETX4} (c) and sd^{58d} (d) mutants. The last line of this construct, ($^{\#}$8-3) rescues both sd^{ETX4} (e) and sd^{58d} (f) wings virtually to wildtype wings. Panel b is a composite of three images.

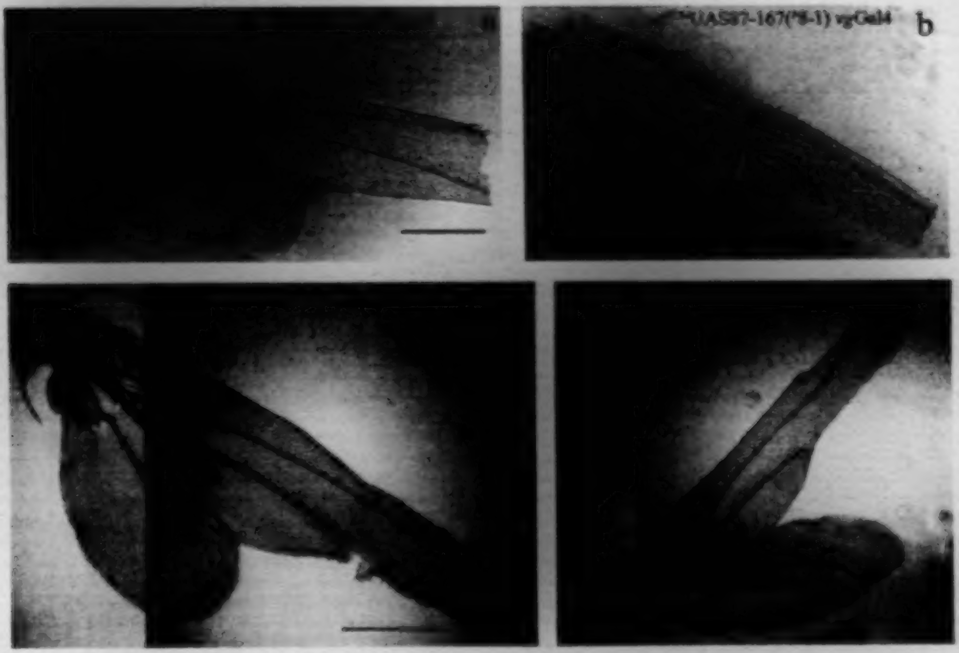

sd^{E3}UASΔ1-87(*7-1) vgGal4
a
c
sd^{E3}UASΔ1-87
(*7-2) vgGal4
d
sd^{58d}UASΔ1-87(*7-2) vgGal4
sd^{E3}UASΔ1-87(*8-3) vgGal4
e
sd^{58d}UASΔ1-87(*8-3) vgGal4
f

3.2-3 Rescue results for transgenic flies expressing Sd containing only the DNA binding domain, and Sd containing the DNA binding domain fused in-frame to the C-terminal domain

To determine if Sd requires Vg to bind certain DNA targets, construct 87-159 which retains only the TEA DNA binding domain (#22-1, #40-1), and construct 87-167 which retains the TEA DNA binding domain including the putative NLS (#8-1, #25-1) were each tested to determine if any effect on wing development would be observed. Thus, these constructs were deleted for the region encoding the portion of Sd that would bind Vg. Expression of both of these constructs caused a dominant negative effect in the sd^{ETX4} background but had no effect in sd^{58d} mutants. In addition, the dominant negative effects associated with expression of these constructs were weak (Figure 3.10a & b). Expression of lines #22-1, #40-1 of 87-159 caused 15% and 43% of the sd^{ETX4} mutants, respectively, to deteriorate to category -4 wings (Figure 3.10a). Expression of both independent lines of 87-167, (#8-1, #25-1) caused 37%, and 18% of the sd^{ETX4} wings to -4 wings, respectively (Figure 3.10b). The rest of the sd^{ETX4} wings were not affected by expression of either construct 87-159 or 87-167.

According to the study done on TEF-1, the C-terminal domain affected TEF-1 binding to DNA targets (Hwang et al., 1993). To determine if this also applies to Sd, construct 87-167 + 345-440 (#23-1) (TEA DNA binding domain containing the putative NLS fused in frame with the C-terminal end of Sd) was generated. Similar to constructs 87-159 and 87-167, upon expression in transgenic flies, it caused a weak dominant negative effect in the sd^{ETX4} background but had no effect in sd^{58d} mutants. Expression of this construct resulted in 48% of the sd^{ETX4} wings being scored as -4 (the rest of the wings were not affected) (Figure 3.10d).

Figure 3.10: The effects of over-expressing the TEA DNA binding domain of Sd on wing development. Transgenics over-expressing the TEA DNA binding domain of Sd produced a weak dominant negative effect in sd^{ETX4} background, and fusing this region to the linker or the C-terminal domain of Sd did not change the rescue outcome. (a) Construct 87-159 (TEA DNA binding domain alone) (#40-1), (b) 87-167 (TEA DNA binding domain including the putative NLS) (#8-1), (c) 88-219 (fusing the linker to the TEA DNA binding domain) (#12-1), and (d) 87-167+345-440 (fusing the TEA DNA binding domain to the C-terminal domain) (#23-1) caused 43%, 37%, 24%, and 48% of the sd^{ETX4} wings respectively to deteriorate to -4 wings whereas the remainder of the sd^{ETX4} wings were not affected. The rescue phenotypes for the other lines of the above constructs were similar and therefore not shown. None of these constructs had an effect in sd^{58d} wings and results are not shown. Panels c and d are composites of two pictures.


a
UAS87-167(*8-1) vgGal4
b

3.2-4 Rescue results for transgenic flies expressing Sd with interruptions or deletions in the DNA binding domain and Sd with the DNA binding domain fused in-frame to the VID

The TEA DNA binding domain of Sd was determined by sequence homology to a region of TEF-1 that interacts with DNA (Campbell et al., 1992). To test if this region of Sd also binds DNA *in vivo* in *Drosophila*, various *sd* constructs deleting the region encoding the TEA DNA binding domain were made. These include six independent lines of Δ1-200 (#5-4, #10-1, #11-2, #15-1, #34-1, and #35-5), three independent lines of Δ88-123 (#2-2, #2-8, and #3-1), two independent lines of Δ124-159 (#6-2, and #13-7), two independent lines of Δ88-159nb (#9-8, and #9-10), and three independent lines of Δ88-159b (#2-1, #7-1, and #38-1). The Δ1-200 construct deletes the region encoding the N-terminal domain along with the entire TEA DNA binding domain of Sd. Expression of this construct resulted in an extreme dominant negative effect (scored as -7) in both mutant backgrounds (Figure 3.7g & h). The wings were reduced to a small outgrowth with prominent necrosis of the wing blade, and all vein patterns were destroyed. Interestingly, when Δ1-200 #15-1 was expressed along with a full length *vg* construct, the dominant negative effect was alleviated in both mutant backgrounds. These wings were scored as a -4 and a -6 in sd^{ETX4} and sd^{58d} mutants, respectively (Figure 3.7i & j). The *sd* constructs Δ88-123, Δ124-159, Δ88-159 delete the first half, the second half, and the entire TEA DNA binding domain of Sd. When expressed in transgenic flies, the various lines of Δ88-123, and Δ124-159 caused both sd^{ETX4} and sd^{58d} mutant flies to produce extremely defective wings (scored as -7 wings) (Figure 3.6e & f, and 3.7a & b). Transgenics of Δ88-159nb (#9-8 and #9-10) resulted in -7 wings in sd^{ETX} mutants while a slightly less severe effect was noted in a sd^{58d} background (75%, and 63% of the sd^{58d}

wings appeared as -7 wings when construct Δ88-159nb #9-8 and #9-10 were expressed respectively, the rest of the wings were not affected) (Figure 3.7c & d). Expression of construct Δ88-159b #2-1, and #38-1 again caused *sd*^{ETX} mutants to produce wings scored at -7, while 42% and 100% of the *sd*^{58d} mutants were scored to -7 wings respectively (Figure 3.7e & f).

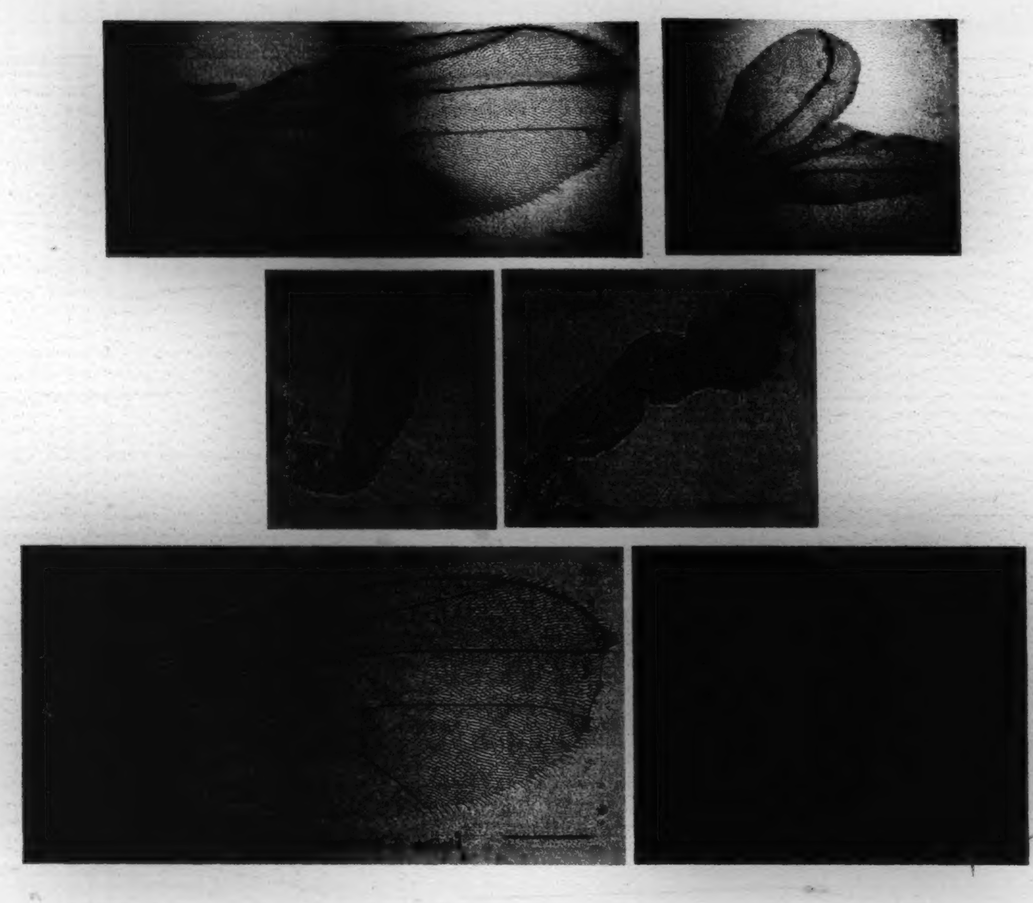
According to Srivastava et al., 2002, only the TEA DNA binding domain of Sd is critical for wing development as long as Vg is already associated with this domain of Sd. To test if this holds true, transgenics of construct 87-167 + 220-344 (#19-1, #28-1) (the TEA DNA binding domain of Sd containing the putative NLS fused in frame with the VID) was generated. Interestingly, both lines of this construct when expressed were unable to behave as wildtype full length Sd, as neither had an effect in *sd*^{ETX4} or *sd*^{58d} mutant backgrounds.

3.2-5 Rescue results of transgenic flies expressing Sd with deletions in the C-terminal domain

There are two major regions of Sd for which no clear functions have yet been identified. The first is the C-terminal domain, the portion of Sd downstream from the VID. To determine if this region functions in wing development, transgenic flies containing a construct Δ345-440 (#31-2, #31-4) that deletes this region were made. It did not have an effect in either wing mutant background. Therefore, to determine the minimal region of the C-terminal region of Sd required for proper wing development, additional constructs (and subsequent transgenics) that sequentially delete the C-terminal domain were generated including: Δ365-440 (#8-1, #24-1), Δ391-440 (#9-1, #9-2, #29-1), Δ416-440 (#9-2, #11-1), and Δ434-440 (#17-1, #21-2) . In addition to further dissecting the C-terminal domain of Sd, constructs Δ416-440, and Δ434-440 also serve to help determine whether the putative finger motif (region 416 to 433) of Sd has any function in wing development (Campbell et al., 1992). Construct Δ416-440 deletes the DNA encoding the C-terminal domain of Sd that includes the putative finger motif, whereas Δ434-440 deletes the DNA encoding the C-terminal domain of Sd up to the finger motif. Flies expressing Δ365-440 (#8-1, #24-1), Δ391-440 (#9-1, #9-2, #29-1), and Δ416-440 (#9-2, #11-1) did not exhibit any effect on wing phenotype in a sd^{ETX4} or a sd^{58d} background. However, construct Δ434-440 #17-1 did rescue sd^{ETX4} mutants to -1 wings, but the rescue effect was much weaker in the sd^{58d} background (-5 wings) (Figure 3.11a & b). The numerical value of -5 represents wings that have the majority of the wing blade missing (Figure 3.3). In contrast, line #21-2 of Δ434-440 exhibited both a rescue in 54% of sd^{ETX4} (-1 wings), and 62% of sd^{58d} wings (-3 to -4 wings), and a dominant negative effect (-7 wings) in both mutants (46% in sd^{ETX4} wings, and 38% in sd^{58d} wings) (Figure 3.11c to f).

As mentioned above, there is a putative finger motif found at the C-terminal end of Sd, located from amino acids 416 to 433 of the protein (Campbell et al., 1992). This motif was deduced from sequence comparison with TEF-1 (Campbell et al., 1992). It is not known whether it has any function in facilitating Sd interaction with DNA targets during wing development. Therefore, transgenics of constructs Δ345-415 (#15-1, #21-1) (deletes the region between the VID and putative finger), and Δ416-433 (#23-1, #24-1, #30-1) (deletes the putative finger) were also generated and assayed for wing rescue. Flies expressing these constructs (Δ345-415, and Δ416-433) were found to have no effect in either mutant background.

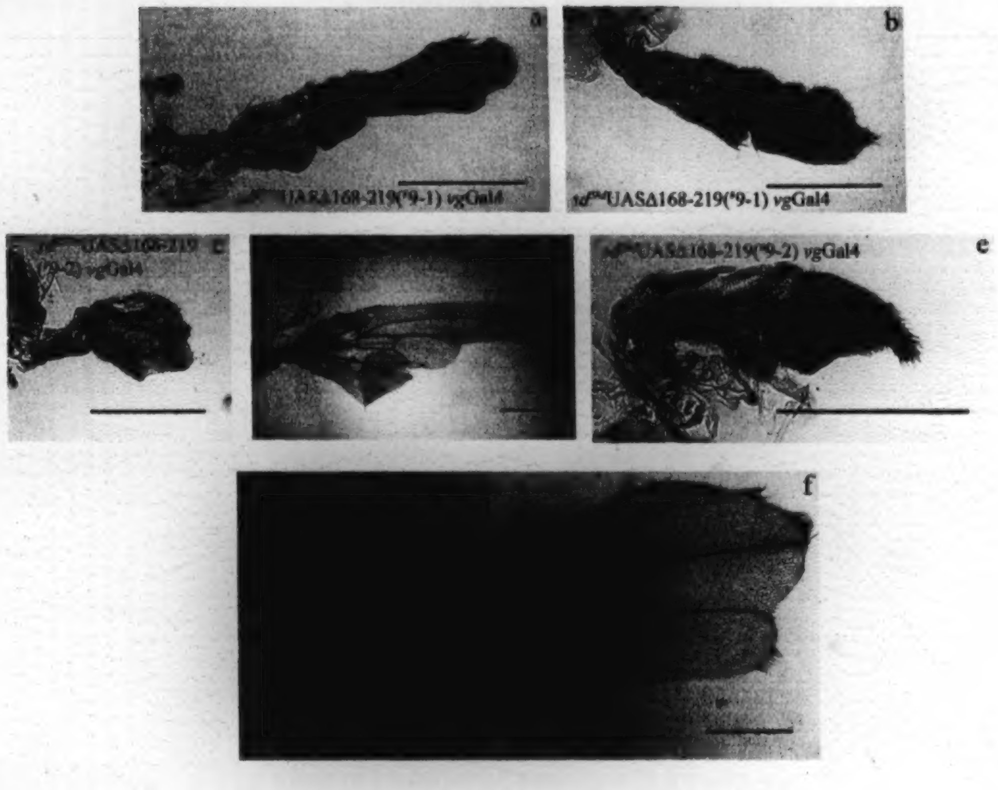
Figure 3.11: The C-terminal domain of Sd is required for its function in wing development. Construct Δ434-440 was able to restore partial function with respect to wing development. Transgenic line #17-1 expressing this construct was able to rescue *sd*^{ETX4} wings to -1 wings (a), but the rescue effect was much weaker (-6 wings) in the *sd*^{58d} background (b). Another independent line of Δ434-440 (#21-2) exhibited a dominant negative effect in 46% of the *sd*^{ETX4} (c) and 38% of the *sd*^{58d} (d) wings. Interestingly, this same transgenic line caused a rescue to -1 wings in the other 54% of the *sd*^{ETX4} flies (e) and between -3 to -4 wings in the other 62% of the *sd*^{58d} (f) flies. Panel a is composed of two images.



3.2-6 Rescue results of transgenic flies expressing Sd with interruptions in the linker domain

A second poorly defined region within the Sd protein is the sequence between the TEA DNA binding domain and the VID (linker region). It is not known whether this region is necessary for *Drosophila* wing development. Therefore, transgenics of constructs Δ160-219nb (#5-1, #6-1, #12-1, #25-1, #36-1), and Δ160-219b (#17-1, #24-1, #25-1) were made and assayed for wing rescue. These two constructs delete this intervening linker region of Sd. Expression of all five independent lines of Δ160-219nb and three independent lines of Δ160-219b produced a strong dominant negative (-7) phenotype in sd^{ETX4} and sd^{58d} mutant flies (Figure 3.6a to d). Two other constructs, Δ168-219 and 168-219R were generated and expressed to measure their ability to influence wing development. Δ168-219 has the linker region deleted but retains the entire putative NLS and an additional four amino acids extending beyond the putative NLS. The Δ168-219R construct has the linker region replaced with a random piece of DNA of equal length (note: this construct also retains the entire putative NLS as does Δ168-219). In theory, both of these constructs should have the ability to enter the nucleus. Two independent lines of Δ 168-219 (#6-1, and #9-1) still exhibited a dominant negative effect when expressed in sd^{ETX4} or sd^{58d} backgrounds resulting in -7 wings (Figure 3.12a & b). However, expression of another independent line of this same construct (#9-2) caused a dominant negative effect in 65% of the sd^{ETX4} flies to (-7 wings), and the other 35% exhibited a weaker dominant negative effect (-4 wings) (Figure 3.12c & d). In the sd^{58d} mutants, expression of this construct resulted in 55% showing a dominant negative effect (-7 wings), while the other 45% exhibited a weak

Figure 3.12: The linker region of Sd has a role in wing development. A transgenic expressing construct Δ168-219 (entire linker region deleted) line #9-1 caused a dominant negative effect in both (a) *sd*^{ETX4} and (b) *sd*^{58d} mutants. The dominant negative phenotype in both mutant backgrounds is similar with line #6-1 of this same construct, thus those results are not shown. (c) However, expression of another independent line of Δ168-219 (#9-2) caused 65% of the *sd*^{ETX4} flies to have a dominant negative effect (-7 wings), (d) and the other 35% a weaker dominant negative phenotype (-4 wings). (e) In the *sd*^{58d} background, it caused 55% of the flies to have a dominant negative effect (-7 wings), (f) and the other 45% a weak rescue at scores between -3 to -4. This rescue result is similar to results obtained with construct 168- 219R (linker domain replaced with a piece of DNA of equal length) as shown in the next figure, suggesting that the linker region does more than serving as a spacer to position the TEA DNA binding domain from the VID. Panel f is a composition of two pictures.


a
UASΔ168-219(*9-1) vgGal4
b
sd UASΔ168-219(*9-1) vgGal4
c
UASΔ168-219
(*9-2) vgGal4
e
UASΔ168-219(*9-2) vgGal4
f

rescue effect (-3 to -4) (Figure 3.12e & f). Expression of the 168-219R construct produced very similar rescue results to Δ168-219. Transgenic flies expressing one independent line of 168-219R ($^{\#}$39-1) resulted in a dominant negative effect (-7 wings) in both mutant backgrounds. Again, expression of another line of 168-219R ($^{\#}$40-1) produced a dominant negative effect in 100% of the *sd*^{ETX4} mutants (-7 wings) but only in 53% of *sd*^{58d} wings, and the other 47% exhibited -3 to -4 wings (Figure 3.13a to e). Since both of these constructs behaved similarly in the rescue assay, this suggests that the linker domain of Sd serves a function during wing development, other than simply acting as a spacer (see discussion for details).

3.2-7 Testing the function of the linker region of Sd and rescue results of transgenic flies expressing Sd constructs 137-219, 88-219, 137-344, & 137-219+345-440.

It is possible that the linker region contains the dimerization domain of Sd. It is known that Sd can interact with itself (Srivastava et al., 2004). To test the possibility that the dimerization domain is within the linker region, a GST pull down experiment was performed to test the ability of Sd Δ168-219 (Sd deleted in the linker region) to bind another full length Sd. The results indicate that Sd Δ168-219 retains its ability to bind another Sd molecule (Figure 3.14) (unpublished results from Hua Deng). Therefore, region 168-219 does not contain the dimerization domain of Sd. In a further attempt to identify a possible function for this domain, construct 137 to 219 ($^{\#}$16-1) (only the linker region is retained) was made and assayed to determine if it had any effect in wing development *in vivo*. This construct was found to have no effect in *sd*^{ETX4} and *sd*^{58d} mutants. It is possible that other regions of Sd are necessary for the function of the linker domain, therefore, transgenics expressing constructs 88-219 ($^{\#}$8-1, $^{\#}$12-1, $^{\#}$22-1), 137-344

Figure 3.13: Flies expressing construct 168-219R (#39-1) have wings exhibiting a dominant negative effect (-7) in both (a) sd^{ETX4} and (b) sd^{58d} backgrounds. (c) Expression of another independent line of this construct (#40-1) causes a dominant negative effect (-7 wings) in sd^{ETX4} mutants. (d) In the sd^{58d} background, 53% of the mutants have a dominant negative effect (-7 wings) and (e) 47% exhibited a weak rescue (-3 to -4 wings). Panel e is composed of two images.

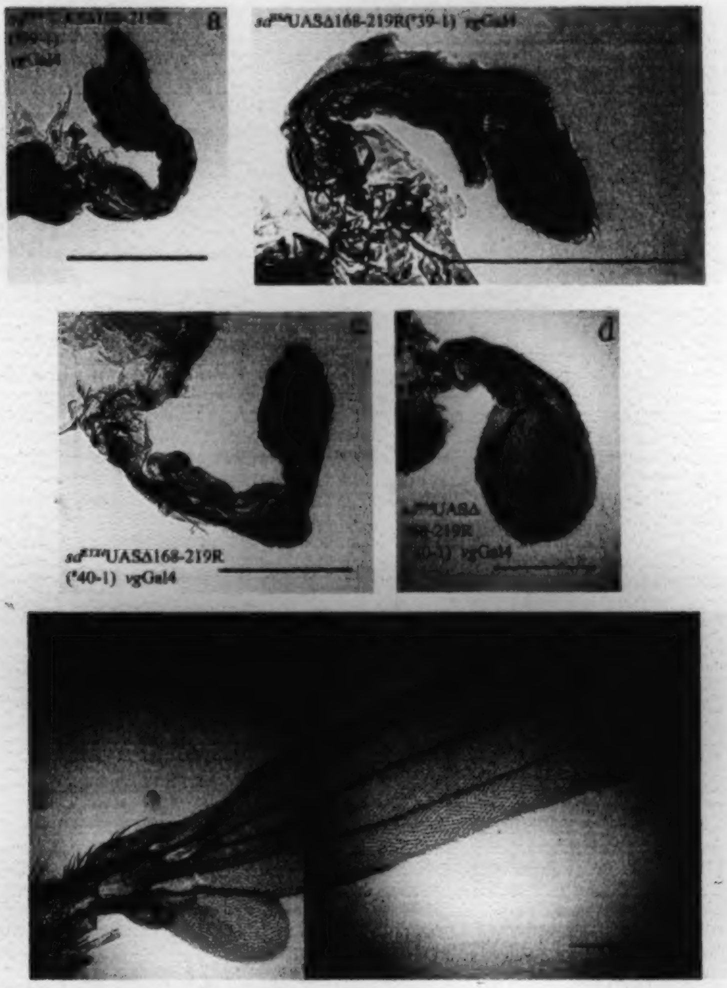
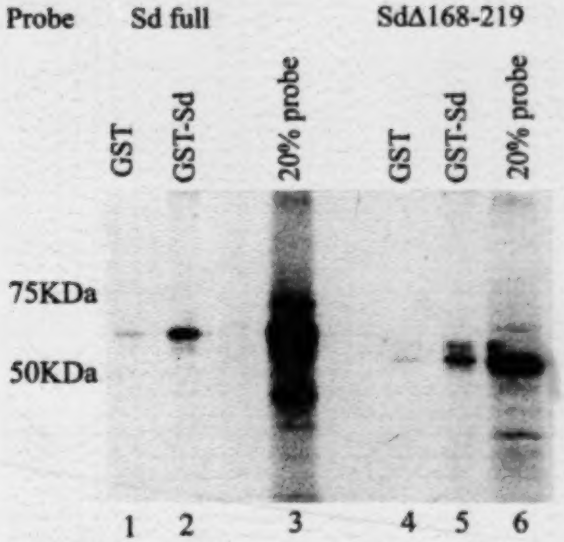

a
(*39-1)
vgGal4
sdUASΔ168-219R(*39-1) vgGal4
c
sdUASΔ168-219R
(*40-1) vgGal4
d
UASΔ
219R
vgGal4

Figure 3.14: The linker region of Sd is not its dimerization domain. Lanes 1 and 4 are GST controls probed with full length Sd and Sd Δ168-219, respectively, showing that GST alone does not bind to either of these proteins. Lane 2 is GST-full length Sd probed with radioactive full length Sd, illustrating that Sd can dimerize with itself. Lane 3 is 20% of the radioactively labeled full length Sd probe used in lanes 1 and 2. Lane 5 is GST-Sd probed with radioactive Sd Δ168-219, indicating that the linker region is not necessary for Sd to interact with itself. Lane 3 is 20 % of the radioactive labeled probe used in lanes 1 and 2. Lane 6 is 20% of the radioactive labeled probe used in lanes 4 and 5. Note the smaller size of the band in lane 5 is consistent with the linker being deleted from Sd.



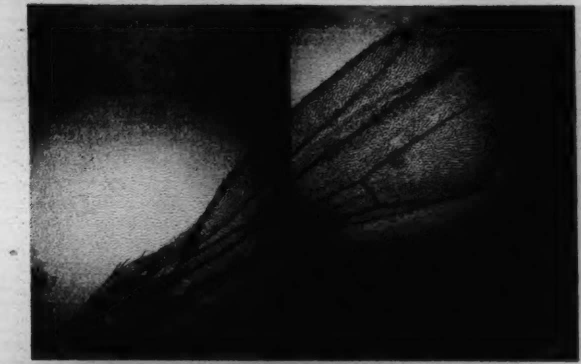
Probe
Sd full
SdΔ168-219
GST
GST-Sd
20% probe
GST
GST-Sd
20% probe
75KDa
50KDa
1
2
3
4
5
6

(#19-1), and 137-219+345-440 (#19-1, #24-1) were also assayed in the rescue experiment. Construct 88-219 consists of the TEA DNA binding domain and the linker sequence, while construct 137-344 retains the linker region and the VID, and construct 137-219+345-440 consists of the linker domain fused in frame with the C-terminal end of Sd downstream from the VID. The three independent transgenic lines of 88-219 (#22-1, #8-1, #12-1) caused 5%, 7%, and 24% of the *sd*^{ETX4} flies to exhibit -4 wings, respectively (Figure 3.10c)(the rest of the wings were not affected). In contrast, neither constructs 137-344 nor 137-219+345-440 had any effect in the two mutant backgrounds.

3.3-8 Results of the rescue assay at 18°C and 29°C

As mentioned previously, the relative levels of Sd and Vg are very critical to proper *Drosophila* wing morphogenesis. Therefore, full length Sd (#34-1), and Δ1-87 (#7-2, and #8-3) were tested in the wing rescue assay at 18°C and 29°C. It is known that the UAS-Gal4 system is more active at 29°C and less active at 18°C compared to room temperature (Brand et al., 1994, Kumar and Moses 2001, Speicher et al., 1994). Presumably, the level of expression of the Sd constructs would be greater at 29°C compared to room temperature, and the level of expression at room temperature would be greater than at 18°C. Interestingly, the dominant negative effects caused by expression of full length Sd line #34-1 and Δ1-87 line #7-2 at room temperature in both the *sd*^{ETX4} and *sd*^{58d} mutants (Figure 3.8c & d, 3.9c & d) were alleviated (-1 wings) when the rescue cross was performed at 18°C (Figure 3.15a to d). In contrast, line #8-3 of Δ1-87 which exhibited a rescue effect at room temperature when expressed (Figure 3.9e & f) caused a dominant negative effect (-7 wings) in both mutants at 29°C (Figure 3.16).

Figure 3.15: The rescue outcomes of transgenics expressing full length Sd and Δ1-87 were changed by shifting the rescue assay to different temperatures. When specific transgenics harboring these constructs were expressed at room temperature, they could cause a dominant negative effect in both *sd*^{ETX4} and *sd*^{58d} wings (Figure 3.8 and Figure 3.9). This effect was alleviated when the rescue cross was performed at 18^{0}C with full length Sd ($^{\#}$34-1) in (a) *sd*^{ETX4} and (b) *sd*^{58d} mutants and (c) Δ1-87 ($^{\#}$7-2) in *sd*^{ETX4} and (d) *sd*^{58d} backgrounds. Panels a and b are composites of two pictures.



UAS-
18°C

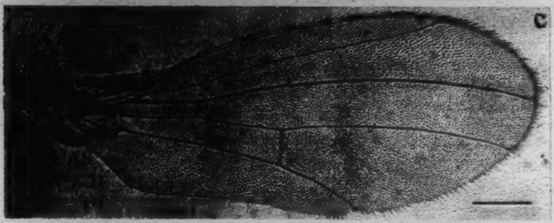
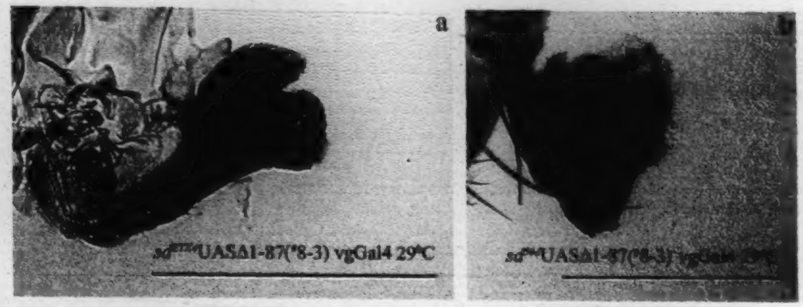

c

Figure 3.16: Expression of construct Δ1-87 at 29^{0}C caused different rescue results compared to the room temperature assay in both wing mutants. The rescue cross of Δ1-87 $^\#$8-3 when done at room temperature rescued both wing mutants (Figure 3.9). However, the rescue effect changed dramatically to a dominant negative effect in both the (a) sd^{ETX4} and (b) sd^{58d} backgrounds at 29^{0}C.


a
sd^{ETX4}UASΔ1-87(*8-3) vgGal4 29°C
b
$sd^{[illegible]}$UASΔ1-87(*8-3) vgGal4 29°C

3.3 Discussion

3.3-1 *sd* expression level is critical for proper wing development

Depending on the respective transgenic strain or the rearing temperature of the flies harboring full length Sd and Δ1-87, different rescue effects can be observed. A possible explanation for this result may be related to the fact that all constructs are randomly integrated into the genome during the injection process. The level of expression of each independent line of any construct will be influenced by the local genomic context. Wing morphogenesis is very sensitive to the relative level of Sd and Vg in the cell (Halder et al., 1998; Simmonds et al., 1998). Therefore, it is possible that full length Sd #18-1 and Δ1-87 #8-3 were able to rescue the two mutants because they allowed the appropriate amount of Sd to be expressed relative to Vg in the cell for the rescue of both mutant wings. However, expression of full length Sd #34-1 and Δ1-87 #7-2 caused a dominant negative effect because they expressed a level of Sd relative to Vg that was not optimal for proper wing morphogenesis. Furthermore, the dominant negative effect caused by expression of line #34-1 was likely due to an over expression of this construct. Evidence for this notion is provided by performing the rescue assay at three temperatures (room temperature, 18°C, and 29°C). For example, expression of full Sd line #34-1 and Δ1-87 #7-2 caused a dominant negative effect in both mutant backgrounds at room temperature (Figure 3.8 and 3.9) whereas at 18°C this dominant negative effect was alleviated, suggesting that when there is a lower level of *sd* expression, the dominant negative phenotype was eliminated (Figure 3.15). In contrast, the rescue effect in both mutants became a dominant negative phenotype when Δ1-87 #8-3 was expressed at 29°C (Figure 3.16), suggesting that the dominant negative effect was due to an over-expression of Sd. This implies that there is an optimal Sd level appropriate for wing rescue. When the Sd protein exceeds this level, a dominant negative phenotype is produced.

For construct Δ1-87 ($^{\#}$7-1), there is variation of rescue outcomes between the two mutant backgrounds. Its expression can cause a dominant negative effect in sd^{ETX4} mutants, yet it rescues sd^{58d} wings (Figure 3.9). Perhaps this transgenic line is expressing a level of Sd that is appropriate for wing rescue in the sd^{58d} background but this level of expression may be too high in sd^{ETX4} mutants, therefore causing a dominant negative phenotype. A possible explanation for the difference in rescue phenotypes between the two mutants can be due to the fact that sd^{58d} is a more severe allele than sd^{ETX4}. Therefore, it likely requires a higher level of Sd to rescue its wing defects. Since sd^{ETX4} is less severe, the same amount of *sd* expression may cause a dominant negative effect.

Because Sd missing the N-terminal 87 amino acids behaves as full length Sd in both mutant backgrounds, this suggests that these 87 amino acids are not vital to the function of the protein with respect to wing development. The N terminal region of Sd is divergent from the human TEF-1 gene family (Deshpande et al., 1997). In addition, no lethal allele of *sd* has been mapped to this region of the protein (Srivastava et al., 2004). In general, Sd deletion constructs that behave similarly to full length Sd indicate that the deleted region is not necessary for wing development. However, when expression of a particular deletion construct does not behave as the full length protein, this indicates that the deleted region may be important in wing development, or at least the missing region results in improper folding of the remainder of the protein and this can impair any function related to wing development.

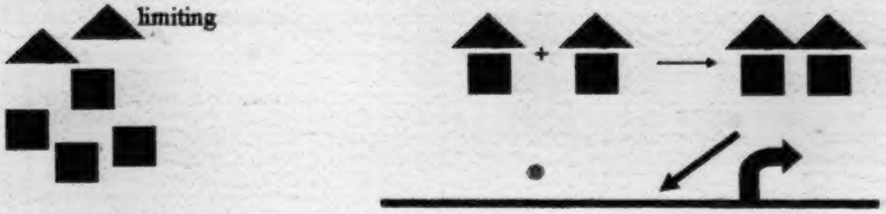
There are several theories to explain how over-expression of Sd can cause a dominant negative effect in the wings. First, there is evidence that Sd by itself binds certain DNA targets termed A sites but when Sd is complexed with Vg it binds different DNA targets termed B sites (Figure 3.1) (Halder and Carroll, 2001). The difference in binding selectivity is thought to be caused by a conformational change in Sd when it interacts with Vg (Halder and Carroll, 2001). It was also hypothesized that while Sd alone can bind A sites, the Sd and Vg proteins need to form a tetramer before binding to B sites (Halder and Carroll, 2001). It is possible that Sd needs to form a dimer with Vg first and then associate with another Sd-Vg dimer to form this tetramer. Consequently, when there is an over-expression of Sd, this skews the optimal relative ratio of Sd and Vg for formation of the Sd-Vg dimers, to the formation of Sd dimers. As previously suggested, Sd can form dimers with itself (Halder and Carroll, 2001; Srivastava et al., 2004). This results in an over-abundance of Sd dimers in the cell that might not be able to form the Sd-Vg tetramer. Thus, an over-expression of full length Sd can cause a dominant negative phenotype in the wing (Figure 3.17a & b). Consistent with this notion, simultaneous expression of *vg* along with full length *sd* line #34-1 was able to alleviate the dominant negative effect caused by over-expression of *sd* alone (Figure 3.8e & f). Another theory is that Sd alone or Sd multimers can act as suppressors of wing target genes when associated with A sites. Therefore, when full length *sd* is over-expressed, there is an increased level of the suppressor in the cell which could contribute to the dominant negative phenotype. It has also been documented that over-expression of

Figure 3.17a and b: Model of how the Sd-Vg complex may activate target gene transcription.

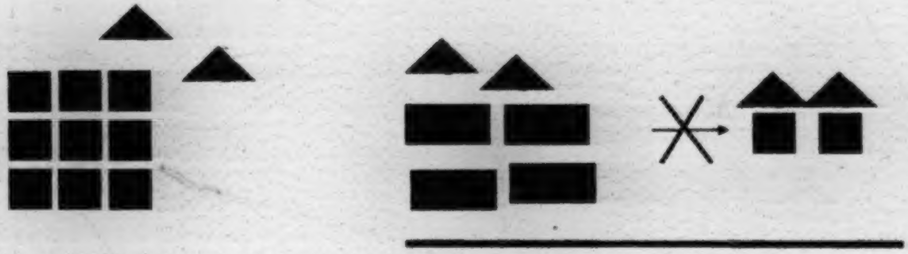
a: In the wildtype situation, the relative concentration of Sd and Vg are optimal for proper wing development. Sd interacts with limiting Vg to form a Sd-Vg dimer which in turn interacts with another Sd-Vg dimer to form a heterotetramer to activate gene transcription.

b: When there is an over-expression of full length Sd in the cell, this can skew the relative ratio of Sd and Vg for formation of Sd-Vg dimers, to formation of the Sd-Sd dimers. These dimers may not be able to form the Sd-Vg tetramer causing a dominant negative phenotype in the wings.

a. Wildtype



b. Over-expression of full length Sd



sd can cause an elevated amount of apoptosis in the wing disc (Liu et al., 2000; Simmonds et al., 1998) and that the pro-apoptotic gene *reaper* can be activated by *sd* expression (Liu et al., 2000). As a result, another factor that could contribute to the dominant negative phenotype is an increased level of apoptosis in the wing disc. Alternatively, Sd has been shown to affect cell growth in the wing blade (Liu et al., 2000). Consequently, the dominant negative phenotype may also be attributed to aberrant cell growth in the wing blade.

3.3-2 *In vivo* evidence to support Vg as the activating component of the Sd-Vg complex and Vg alters Sd DNA binding specificity

Constructs that are interrupted or deleted in the VID had no effect in sd^{ETX4} or sd^{58d} mutants. This supports the idea that Vg is the activating component of the Sd-Vg complex (Simmonds et al., 1998) and it also supports the theory that Vg alters Sd DNA binding specificity from A sites to B sites (Halder and Carroll, 2001). When the VID is interrupted, Vg can no longer associate with Sd. Consequently, the activating component of the Sd-Vg complex is missing, rendering the complex unable to function in wing development, and hence have no effect in the rescue assay. If Sd does not require Vg for it to interact with target sequences, Sd with its TEA DNA binding domain intact, but interrupted in the VID, should retain an ability to bind DNA targets. Hypothetically, this will result in a dominant negative phenotype since these mutant Sd molecules lacking the ability to interact with Vg (cannot activate target gene transcription), can still compete with endogenous Sd for DNA target sequences. However, this is not the observed result and suggests that Sd requires Vg to somehow alter its DNA binding specificity. In addition, constructs 88-159 (TEA DNA binding domain only) and 88-167 (TEA DNA binding domain with four amino acids extending beyond the putative NLS) only caused a weak dominant negative phenotype in a portion of sd^{ETX4} mutants (Figure 3.10a & b) which indicates that the TEA DNA binding domain alone can not bind DNA targets efficiently. Nevertheless, this inefficient binding retains partial capacity to out-compete endogenous Sd for the same DNA targets as the Sd-Vg tetramer, thus causing a weak dominant negative phenotype. The C-terminal domain was also fused to the TEA DNA binding domain of Sd to determine its effects in the rescue assay in comparison to expression of the DNA binding domain only. Addition of the C-terminal domain was unable to change the weak dominant negative effect (Figure 3.10d), suggesting that the C-terminal domain does not affect Sd binding affinity to target sequences. However, it is possible that the C-terminal region affects another region of Sd that modulates binding affinity of the TEA DNA binding domain, as in TEF-1 (Hwang et al., 1993).

The previous paragraph suggests that when Sd cannot associate with Vg, it cannot recognize and/or bind its proper target sites and has no effect in the rescue assay. It is not known how Vg alters Sd DNA-binding specificity. It is hypothesized that Vg may cause a conformational change in Sd such that the TEA DNA binding domain loses its ability to associate with A sites while enhancing its ability to interact with B sites (Halder and Carroll, 2001). Alternatively, Vg interaction with Sd can specifically enhance its affinity for B sites (Halder and Carroll, 2001). Another possibility is that Vg interaction with Sd may allow post translational modifications such as phosphorylation within the TEA DNA binding domain such that its affinity for B sites increases. There are potential phosphorylation sites found in the TEA DNA binding domain of Sd (Srivastava et al., 2004). In addition, the observation that expression of constructs interrupted in the VID have no effect in the rescue assay argue against Sd acting as a suppressor when it is not associated with Vg. If it was true, a wing phenotype should be expected, since Sd interrupted in the VID cannot associate with Vg and by default should behave as a suppressor. However, the suppressor effect would be abolished if the VID is needed for suppressor function.

3.3-3 The entire VID and the C-terminal domain of Sd are required for a stable Sd-Vg interaction

What other function(s) could the C-terminal portion of Sd be performing? A possible answer comes from construct Δ345-440. This construct is deleted for the entire C-terminal end of Sd up to the VID, but in theory it should still be able to bind both DNA targets and Vg, to rescue or cause a dominant negative phenotype, depending on the expression level of the protein. The lack of effect imposed by this construct suggests that the C-terminal region of Sd is important for wing development. This region is likely to be necessary for Vg to recognize Sd and/or for Sd-Vg complex stabilization *in vivo* with respect to wing development. When Sd is unable to form a stable association with Vg, it cannot have an effect on the wings, for reasons suggested previously. Wing imaginal discs from third instar larvae harboring the lethal allele sd^{68L} (with a base substitution of tyrosine to asparagine mapping ten amino acids beyond the VID) shows considerable cytoplasmic localization of Vg *in vivo* whereas *in vitro* this version of Sd can still bind Vg (Srivastava et al., 2004). Similarly, it was found that TEF-l retaining only the entire VID and the C-terminal domain can interact with Vgl-1 *in vivo* in a Yeast two hybrid assay (Maeda et al., 2002).

However, there are some findings that are inconsistent with the above theory. First, an *in vitro* biochemical assay found that TEF-1 truncated at the C-terminal domain was able to bind Vgl-1 (Vaudin et al., 1999). A possible explanation for the discrepancy is that the two experiments were conducted in two different environments. The wing rescue assays allowed Sd to be in its native environment where cellular processes such as post translational modification (e.g. phosphorylation) and/or the cellular context itself can affect or set limits on the ability of Sd to bind its partner. In an *in vitro* assay, the cellular environment is absent; only an incomplete supply of biological factors can affect Sd's ability to interact with its partner. Therefore, even though the C-terminal domain of Sd was interrupted, it could still associate with Vg *in vitro*. Second, a lethal allele of *sd* (*sd*^{11L}) with a base substitution (histidine to leucine) at residue 433 of Sd was able to complement the *sd*^{ETX4} wing phenotype (Srivastava et al., 2004). Presumably this result suggests that this aberrant Sd was able to form a stable interaction with Vg to activate transcription of downstream wing target genes. A possible rationalization to explain this discrepancy is that it is the folding and conformation of the entire VID and C-terminal domain that somehow allows a stable interaction between Sd and Vg. Perhaps, the base substitution found in *sd*^{11L} does not alter the overall conformation of the VID and C-terminal domain whereas the base substitution involving *sd*^{68L} does. Finally, it can also be entertained that the amino acid substitution in *sd*^{11L} is not important for Vg to recognize and or bind Sd whereas the mutation found in *sd*^{68L} is.

Further C-terminal deletion constructs including Δ365-440, Δ391-440, and Δ416-440 had no effect in the wing rescue assay. This is consistent with the notion that the entire VID and C-terminal domain of Sd are required for a stable Sd-Vg complex.

However, expression of construct Δ434-440 with only the last seven C-terminal amino acids deleted was able to cause either a rescue or a dominant negative phenotype in both mutant backgrounds (Figure 3.11). This is reminiscent of the situation when full length *sd* was expressed. However, the rescue caused by Δ434-440 was not as complete as with full length Sd, especially in the sd^{58d} background (Figure 3.11b & f). There are several possible explanations for this observation. It may be that the level of expression of Δ434-440 was not optimal for a full rescue. It is also possible that the Δ434-440 truncation in Sd can render it less stable than the full length protein itself, even though this deletion construct is known to be expressed. Therefore, the decreased stability may compromise its ability to fully rescue sd^{ETX4} and sd^{58d} mutants. Alternatively, the entire C-terminal domain may be necessary for stable Sd-Vg interaction *in vivo*. Evidence for this possibility is that expression of construct 137-344 (the putative NLS and the linker region fused in frame with the VID) did not have any effect in either mutant background. If only the VID is needed for Sd-Vg stable interaction, this construct without the ability to bind DNA should titrate limiting Vg away from endogenous Sd to cause a dominant negative phenotype. In addition, transgenic flies expressing construct Δ1-200, that retains the VID and the entire C-terminal domain of Sd, was able to cause a dominant negative effect in both mutants, possibly by titrating limiting Vg (Figure 3.7g & h).

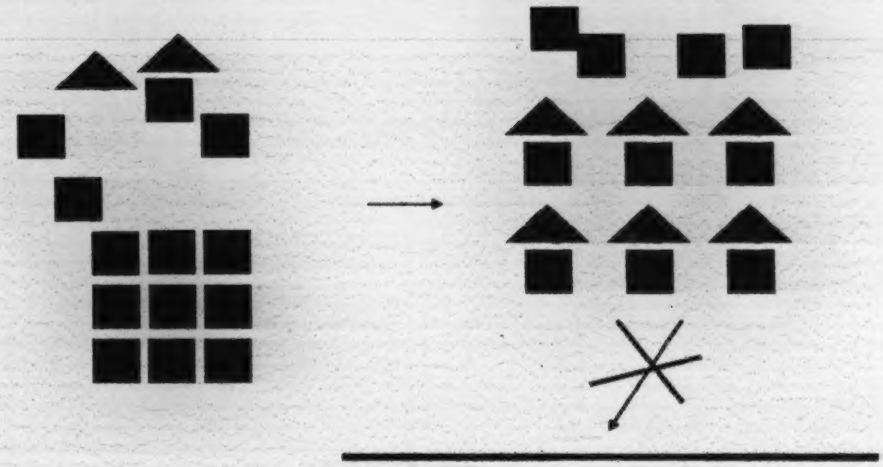
It appears that the entire C-terminal domain of Sd may be required for Vg interaction and consequently also the activation function of the Sd-Vg complex. It has already been shown that over-expressing Vg in a sd^{58d} mutant resulted in considerable cytoplasmic localization of Vg (Srivastava et al., 2002). Therefore, to verify that the C-terminal domain is required for a stable Sd-Vg complex, Vg antibody staining can be performed in a sd^{58d} mutant over-expressing both Vg and Sd with C-terminal end deletions. The result from this analysis could then be compared with the pattern of Vg staining in a sd^{58d} mutant over-expressing both Vg and full length Sd #18-1. If the Vg staining pattern is diffuse and cytoplasmic in the former scenario but punctate for the latter, this would support the theory that the entire C-terminal domain along with the VID is required for a stable Sd-Vg complex *in vivo*. These *in vivo* results could then be confirmed *in vitro*, by testing the ability of *sd* constructs with C-terminal deletions to bind Vg via Far Western blot analysis. Furthermore, additional sequential deletions of the C-terminal domain of construct Δ1-200 can be made to determine the minimum region necessary to cause a dominant negative effect in the wings. This will be indicative of a stable Sd-Vg interaction, and defines the minimum region required for Sd and Vg to form a stable complex.

The C-terminal region of Sd almost certainly has other roles in development in addition to being necessary for the stability of the Sd-Vg complex. It has been found that there is a putative finger motif located at amino acids 416 to 433 of Sd, as deduced from sequence comparison with TEF-1 (Campbell et al., 1992). Expression of Sd constructs deleted for the entire motif (Δ416-433) and deleted for the region between the VID and the motif (Δ345-415) did not have any effect in either sd^{ETX4} or sd^{58d} mutant backgrounds. Unfortunately, the lack of effect does not reveal whether the putative finger motif has any role in wing development because this overlaps with the region predicted to be necessary for Vg interaction. However, these results are consistent with the notion that the C-terminal domain is necessary for stable Sd-Vg interaction. If these regions were not needed for Vg interaction, constructs Δ416-433 and Δ345-415 should associate with Vg to either rescue or cause a dominant negative effect in the wings.

3.3-4 The TEA DNA binding domain of Sd is likely to bind DNA targets *in vivo*

The role of the TEA DNA binding domain of Sd was also investigated. Expression of Sd constructs interrupted in this region caused a dominant negative effect in both sd^{ETX4} and sd^{58d} mutant backgrounds (Figure 3.6e & f, 3.7a to h). It is likely that these aberrant Sd molecules can still associate with limiting Vg in the cell, yet cannot bind DNA targets. Therefore, these mutant Sd proteins can titrate Vg from endogenous Sd in the cell and thus cause a dominant negative phenotype (Figure 3.18). To support this theory, when Sd Δ1-200 was over-expressed simultaneously with full length Vg, the dominant negative phenotype seen for both mutants was alleviated (Figure 3.7i & j). Construct Δ88-123 has the first half of the TEA DNA binding domain including helix 1 deleted, while helix 2 and 3 are intact. Expression of this construct still caused a dominant negative phenotype, which seems to suggest that *in vivo* Sd without helix 1 in the TEA DNA binding domain cannot associate with DNA targets even though helix 2 and 3 are intact. This is in agreement with the finding that helix 1 of TEF-1 is necessary for sequence specific DNA binding and recognition of DNA targets *in vitro* (Hwang et al., 1993). The dominant negative phenotype caused by expression of construct Δ124-159, with a deletion of helix 2 and 3, suggests that it cannot bind to its DNA target sequences either. Therefore, this supports the previous finding that helix 3 of TEF-1 is also critical for DNA binding (Hwang et al., 1993).

Figure 3.18: A model to explain the dominant negative effects caused by *sd* constructs interrupted in the TEA DNA binding domain. When these aberrant Sd proteins (green) are over-expressed, they may out-compete the endogenous Sd (purple) for limiting Vg. However, the mutant Sd-Vg complex cannot bind DNA targets (the TEA DNA binding domain interrupted in Sd) and causes a dominant negative phenotype in the wings.



3.3-5 The linker region of Sd serves an important yet unknown role in wing development

The function of the linker region of Sd remains unclear. Expression of constructs deleted or replaced in the linker domain caused a dominant negative effect in transgenic flies in both sd^{ETX4} and sd^{58d} mutant backgrounds (Figure 3.6a to d). This indicates that the products encoded by these constructs can still bind Vg, and potentially out compete endogenous Sd for limiting Vg, thus causing a dominant negative effect. Therefore, one can infer that the linker region is not necessary for Vg interaction since a phenotypic effect is still produced. There are at least three possible explanations to explain the dominant negative phenotype caused by these constructs. First, this region of Sd may actually be important for wing development. Second, the last four amino acids of the putative NLS (Srivastava et al., 2004) are deleted in these constructs. The proteins encoded by these constructs can bind and titrate limiting Vg since their VID is still intact but they are unable to enter the nucleus due to the interruption in their NLS. Thirdly, this region of Sd can simply act as a spacer to allow proper folding and positioning of the TEA DNA binding domain with the VID. Perhaps when the DNA binding domain and the VID are juxtaposed to each other there is steric hindrance, which inhibits the protein from functioning properly. For example, Vg may not interact with Sd efficiently when Sd is associated with its DNA target. Alternatively, the proper orientation of the domains of Sd may also be affected when the DNA binding domain is positioned next to the VID, rendering it unable to carry out its wildtype function.

The theory that disruption of the putative NLS caused the dominant negative effect can be discounted. Expression of construct Δ168-219 missing the linker region but with the entire putative NLS intact, still caused a dominant negative phenotype in sd^{ETX4}

and some of the sd^{58d} mutants (Figure 3.12). If disruption of the putative NLS was the main cause of the dominant negative effect in transgenic flies expressing construct Δ160-219, expression of construct Δ168-219 containing the entire putative NLS should have behaved as full length Sd. It is also unlikely that the main role of the linker region is to serve as a spacer for the TEA DNA binding domain and the VID. Expression of both constructs Δ168-219 (Figure 3.12) and 168-219R (linker replaced with a piece of DNA of equal length with the entire putative NLS intact) (Figure 3.13) cause a similar rescue result in both mutant backgrounds. This suggests that the linker domain has a function in wing development, otherwise expression of construct Δ168-219R should result in a similar rescue outcome as expression of full length Sd while expression of Δ168-219 would not.

In addition to causing a dominant negative effect in sd^{ETX4} and sd^{58d} mutants, expression of constructs Δ168-219 and 168-219R also caused a weak rescue in some of the sd^{58d} mutants. It remains unclear why expression of constructs Δ168-219 and Δ168-218R could partially rescue some of the sd^{58d} but not sd^{ETX4} mutants. It is possible that the weak rescue indicates that Sd without the linker domain can retain sufficient function of the Sd protein to partially rescue sd^{58d} but not sd^{ETX4} wings. The incomplete rescue can be attributed to the loss of the unknown function(s) provided by the linker domain. Alternatively, it is also possible that the incomplete rescue is due to Sd protein with the linker region missing or replaced being less stable than full length Sd, as previously suggested with construct Δ434-440. However, this explanation seems unlikely since expression of constructs Δ168-219 and Δ168-218R were not able to provide any degree of rescue in sd^{ETX4} mutants.

Initially, it was speculated that the function of the linker domain is to dimerize with another Sd molecule. This could explain the dominant negative phenotype caused by expression of both constructs Δ168-219 and Δ168-219R. It is possible that when region 168-219 is missing or replaced, the two Sd molecules in the Sd-Vg tetramer can not associate with each other as efficiently as full length Sd. This could lead to a decreased ability of the mutant complex to activate target gene transcription. This mutant Sd-Vg tetramer, with a compromised ability to activate transcription of target genes, could then out-compete endogenous Sd-Vg for target sequences and cause a dominant negative phenotype. However, this idea can now be discounted since the protein encoded by construct Δ168-219 was found to retain the ability to associate with another Sd molecule in an *in vitro* assay (Figure 3.14, unpublished work by Hua Deng). In addition, expression of the construct containing just the region from 137 to 219 (linker domain) of Sd had no effect in either sd^{ETX4} or sd^{58d} background in the rescue assay, which also indicates that this region does not associate with another Sd molecule. If this region did dimerize with another Sd *in vivo*, a dominant negative effect would be expected for reasons suggested previously. Therefore, the function of region 168 to 219 remains unknown and the dimerization domain of Sd also remains elusive. Far Western blot analysis or GST pull down experiments can be used to test various Sd deletion constructs to determine which region is required for Sd dimerization.

Other regions of the Sd protein including the TEA DNA binding domain, the VID, and the C-terminal domain were also tested to determine if any of these regions could affect the function of the linker domain *in vivo*. When the linker region was fused with the VID or the C-terminal domain, expression of these constructs had no effect on wing development. Expression of a construct with the TEA DNA binding domain fused to the linker had a weak dominant negative effect in a small portion of the *sd*^{ETX} mutants (Figure 3.10c), reminiscent of the situation when the TEA DNA binding domain alone was assayed. Therefore, addition of the TEA DNA binding domain to the linker did not have any significant effect on the rescue outcome. Thus, it is likely that none of these regions alone have any effect on the linker sequence. Perhaps, the function of the linker region involves the entire Sd protein except for the N-terminal 87 amino acids, which was suggested earlier to have no function in wing development. It is possible that the linker domain is required to mediate Sd binding to DNA and its ability to form a stable interaction with Vg. Alternatively, the unknown function of the linker domain may be required after Sd is bound to DNA and complexed to Vg.

The function of the linker region remains unknown and speculative at best. However, this region of Sd is unlikely to be required for a stable Sd-Vg interaction, since expression of all constructs with the linker domain interrupted or replaced were able to cause a dominant negative effect in both mutants, which is indicative of limiting Vg being titrated from endogenous Sd. A possible function of this region could involve DNA binding affinity of the Sd protein. It is known that there are regions of Sd outside of the TEA DNA binding domain, such as the C-terminal region, that restrict its binding affinity to certain DNA sequences (Halder and Carroll, 2001; Hwang et al., 1993). In an *in vitro* assay, the TEA DNA binding domain alone was able to bind DNA targets that the full length protein did not interact with (Halder and Carroll, 2001). This could explain why expression of Sd deleted or replaced in this region can cause a dominant negative effect in the *sd*^{ETX4} and *sd*^{58d} mutants. When the DNA binding regulatory region is interrupted, Sd is less able to recognize or bind target sequences. Perhaps when proteins encoded by constructs Δ160-219, Δ168-219, and Δ160-219R are associated with Vg, they cannot bind target sequences as efficiently as the wildtype Sd-Vg complex. Once again, the mutant Sd could bind and titrate Vg from endogenous Sd. This mutant Sd may have a decreased ability to activate target gene transcription, since it cannot form a stable association with its DNA targets, thereby causing a dominant negative phenotype in the wings. It is also possible that the actual sequence within the linker domain is required for the proper folding, conformation, or orientation of the protein such that when Sd is associated with Vg, it can form a stable association with its DNA targets.

Another possibility is that the linker region is the binding or recognition site for other co-activators such as CBP (cAMP-responsive element binding (CREB)-binding protein) in order for the Sd-Vg complex to activate gene transcription properly. Sd has been found to bind CBP (Guss et al., 2001). Therefore, proteins produced by expressing constructs Δ160-219, Δ168-219, and Δ160-219R interrupted in the linker region might not be able to recruit or interact with these co-activators as efficiently as the wildtype Sd-Vg complex. Similar to the explanation above, these mutant Sd proteins could still bind and titrate Vg from endogenous Sd but lack the ability to associate with the appropriate co-activators, thereby causing a dominant negative phenotype in the wings. One way to test this hypothesis is to determine whether Sd deleted in the linker region has the ability to bind CBP using *in vitro* assays such as GST pull down or Far Western blot experiments. Interestingly, the linker region between Sd and TEF-1 is not conserved (Deshpande et al., 1997), so maybe sequence diversity is required for different co-activators to recognize and bind this region.

3.3-6 Possible theories to explain puzzling rescue results

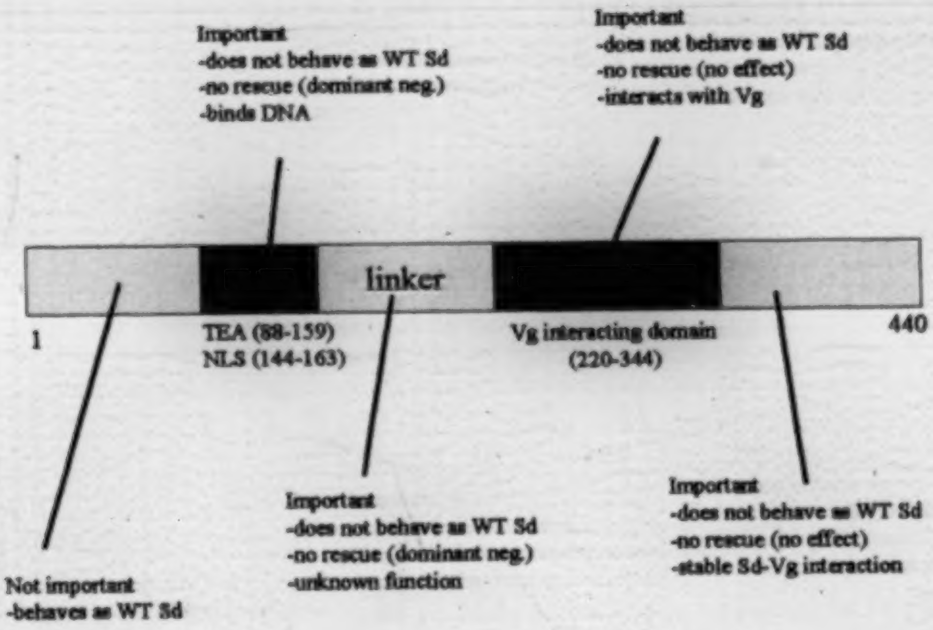
One puzzling result that has not been resolved is why would a construct encoding amino acids 63 to 211 of Sd (contains the entire TEA DNA binding domain, putative NLS, and majority of the linker region) fused in frame with full length Vg be able to rescue both sd^{ETX4} and sd^{58d} mutants (Srivastava et al., 2002). Results presented so far suggest that the entire Sd protein except for the N-terminal 87 amino acids is required for its proper function in wing development. A possible explanation is that since Vg is now already fused to Sd, the role provided by the VID and C-terminal domain can be eliminated. In addition, since only eight amino acids of the linker domain are deleted from the Sd portion of the fusion construct, this may not have any effect on the unknown function of the linker domain. Consequently, the fusion construct was able to behave as full length Sd and rescue the two wing mutants.

In the wing rescue assay, expression of a number of constructs (87-159, 87-167, 88-219, and 87-168 + 345-440) caused an effect in sd^{ETX4} but not in sd^{58d} wings. A possible reason could be that since sd^{58d} is more severe than sd^{ETX4} with respect to wing phenotype, any subtle changes in wing phenotype will be harder to detect. As previously mentioned, expression of the above constructs caused a weak dominant negative effect in sd^{ETX4} wings, therefore these subtle changes to the wing blade might not be apparent in a sd^{58d} background. Alternatively, sd^{ETX4} likely has a cellular level of Sd closer to the wildtype level than sd^{58d} mutants has. Assuming that a level of Sd lower or higher than that in the wildtype situation causes wing malformation, it would take more Sd in the sd^{58d} mutants to reach the threshold level where it would impose a dominant negative effect in the wings. In some cases only a portion of the sd^{ETX4} wings is affected, or one independent transgenic line of a particular construct was able to cause both a rescue and a dominant negative effect in both mutant backgrounds. First, this suggests that proper wing development is extremely sensitive to the level of Sd. Even a slight variation in its expression level between individual flies is enough to change the rescue outcome. The individual differences in expression level between siblings could be attributed to multiple inserts in certain transgenic strains. This could be tested via Southern blot analysis. Environmental factors such as crowding may also affect the level of Gal4 protein being expressed in different flies, which in turn can alter the expression level of the transgene within each organism. This same explanation can be extended to Sd Δ88-159nb, Δ88-159b, Δ434-440, Δ168-219, and Δ168-219R where the same transgenic line of these constructs is capable of producing different rescue outcomes in the two wing mutants.

3.3-7 Model of how the Sd-Vg complex functions to activate wing transcription

In wildtype flies, establishment and maintenance of the relative levels of Sd and Vg are vital for proper wing development (Simmonds et al., 1998). There is a narrow range where the level of Sd can promote proper wing morphogenesis, a level below or above this will cause malformation of the wing. This optimal range of Sd for proper function might be related to how the Sd-Vg tetramer is formed. It is hypothesized that Sd first associates with Vg, and this dimer can then associate with another Sd-Vg dimer. The rescue results suggest that the N-terminal 87 amino acids of Sd are not important for wing development. However, the rest of the protein is needed for Sd to function properly in wing morphogenesis since deletion of any other region of Sd rendered it unable to function as the full length protein in the rescue assay. The TEA DNA binding domain has been localized to lie within residues 88 to 159 of the protein and is required for its interaction with DNA. Amino acids 220-344 of Sd are required for Vg to associate with Sd, as previously defined (Vaudin et al., 1999). However, the entire C-terminal domain may also be necessary for a stable Sd-Vg interaction. In addition, Sd requires Vg to bring about some form of change in itself so it can interact with target sites (Halder and Carroll, 2001). The linker region is also needed for Sd to function properly, but the function of this domain remains elusive. What is known is that this region is not necessary for a stable Sd-Vg interaction. It is possible that this region regulates Sd binding to DNA target sequences (Hwang et al., 1993) or that this region is the binding or recognition site for other co-activators in order for the Sd-Vg complex to activate transcription properly, and finally this domain may be required for the proper folding of the protein. A summary of the functional domains of Sd is in Figure 3.19.

Figure 3.19: Summary of the functional domains of Sd. The red line points to the region that is not necessary for wing development, whereas the blue lines indicate regions that are functionally important. Below each functional region is a summary of its effect when interrupted in the rescue assay in comparison to full length Sd, and its possible role in wing development. Abbreviation: WT;wildtype.


Important
-does not behave as WT Sd
-no rescue (dominant neg.)
-binds DNA
Important
-does not behave as WT Sd
-no rescue (no effect)
-interacts with Vg
linker
1
TEA (88-159)
NLS (144-163)
Vg interacting domain
(220-344)
440
Important
-does not behave as WT Sd
-no rescue (dominant neg.)
-unknown function
Important
-does not behave as WT Sd
-no rescue (no effect)
-stable Sd-Vg interaction
Not important
-behaves as WT Sd

Chapter 4: An *in vivo* assay for the regions of Sd that can induce endogenous *sd* expression

4.1 Introduction

There is an inter-dependent functional relationship between Sd and Vg in *Drosophila* wing development (Halder et al., 1998, MacKay et al., 2003; Paumard-Rigal et al., 1998; Simmonds et al., 1998; Varadarajan and Raghavan, 1999; Williams et al., 1993). Some loss of function alleles of both *sd* and *vg* show similar wing phenotypes (Campbell et al., 1992; Campbell et al., 1991; Williams et al., 1993). Proper *vg* expression is absent in *sd* mutants and vice versa (Williams et al., 1993). Both *sd* and *vg* have very similar patterns of expression in the third instar wing disc (Williams et al., 1993). It has been suggested that expression of *sd* and *vg* in the wing disc are co-dependent (Williams et al., 1993). The autoregulation of Vg requires Sd (Halder et al., 1998b; Simmonds et al., 1998), and Vg can cause ectopic expression of endogenous *vg* (Paumard-Rigal et al., 1998). Ectopic expression of *vg* in the eye-antennal disc (driven with *ptc*-Gal4) was able to activate transcription of the endogenous *vg* gene in this same tissue (Paumard-Rigal et al., 1998). It has also been demonstrated that Vg can induce ectopic expression of Sd (MacKay et al., 2003; Simmonds et al., 1998). This was shown by the activation of the *sd-lac*Z reporter when *vg* expression was directed along the A/P boundary of the wing disc with a *ptc*-Gal4 driver (MacKay et al., 2003; Paumard-Rigal et al., 1998; Simmonds et al., 1998). This is in agreement with the ability of Vg to induce ectopic *vg* expression, since Sd is required for *vg* autoregulation. Interestingly, Sd can also induce ectopic expression of endogenous *sd* (Varadarajan and Vijay Raghavan, 1999). For example, when *sd* expression is targeted to the A/P boundary of the wing disc by *ptc*-GAL4, ectopic expression of endogenous *sd* was also detected along the A/P boundary of the disc (Varadarajan and Vijay Raghavan, 1999). However, it seems that Vg can cause ectopic expression of *sd* in tissues outside of the wing disc, whereas Sd can only cause ectopic expression of *vg* in wing derived tissues (Kim et al., 1996; Varadarajan and Vijay Raghavan, 1999).

It has been proposed that the *sd* promoter itself is a target of the Sd-Vg complex (Simmonds et al., 1998). In addition, Sd binding sites have been found in the *vg*QE (Guss et al., 2001). Furthermore, the Sd-Vg complex can activate the *vg*BE and *vg*QE in *Drosophila* S2 cells (Halder et al., 1998b). As suggested above, full length Sd has the ability to ectopically induce expression of *sd*. Nevertheless, it is not known which regions of the protein are important for this process. Consequently, regions of Sd necessary for inducing expression of endogenous *sd* were examined *in vivo*. In addition to determining region(s) of Sd important for self regulation, this investigation will further dissect Sd functionally.

4.2 Results:

4.2-1 Expression of full length Sd (#34-1) and Δ1-87 can induce ectopic endogenous *sd* expression

To determine which regions of Sd are necessary to cause ectopic endogenous *sd* expression, an ectopic expression assay was carried out as described in Materials and Methods section 2.17. At least one independent transgenic line from all the Sd constructs tested in the wing rescue assay (Table 3.1a, b, & c) was also tested for any ability to induce ectopic expression of the endogenous *sd* allele. As described in the results section of chapter 3, these constructs were first tested to ensure that they are transcribed or translated. Therefore, any lack of ectopic expression is not due to lack of expression of the respective transgene. See Table 4.1a & b for the respective line(s) tested for each construct, and a summary of the ectopic expression results.

At room temperature, all three independent transgenic lines of Sd Δ1-87 (#7-1, #7-2, and #8-3) can induce ectopic expression of *sd* (Figure 4.1a). However, one transgenic line encoding full length Sd (#34-1) can induce *sd* expression (Figure 4.1b), whereas another line encoding full length Sd (#18-1) cannot. To determine whether various temperatures can affect the ability of these constructs to induce ectopic endogenous *sd* expression, these assays were repeated at 18^{0}C, and 29^{0}C. The ectopic *sd* expression caused by Δ1-87 transgenic lines (#7-1, #7-2, and #8-3) and full length Sd (#34-1) was virtually eliminated at 18^{0}C compared to room temperature expression (Figure 4.1e & f). At 29^{0}C, all larvae expressing full length Sd (#34-1), and Δ1-87 (#7-1, #7-2, and #8-3) died, whereas expression of full length Sd (#18-1) still did not cause ectopic expression of endogenous *sd*. As a negative control for the expression essay, pUAST alone was also tested and was found unable to cause ectopic expression of *sd* (Figure 4.1d). None of the remaining *sd* constructs assayed were capable of inducing ectopic *sd* expression at room temperature. However, expression of two of the constructs produced unexpected results. Surprisingly, when transgenic flies harboring constructs Δ168-219 and Δ168-219R were expressed via the *ptc*Gal4 driver, they caused a lethal phenotype. To be more specific, expression of Δ168-219 (#9-1), and Δ168-219R (#39-1) caused a larval lethal effect with no survivors reaching beyond the third instar stage, while expression of Δ168-219 (#6-1), and Δ168-219R (#40-1) caused a pupal lethal effect at room temperature (Figure 4.2) with no survivors emerging from the pupal case.

Table 4.1a Summary of the ectopic expression assay

Constructs	Domain(s) interrupted	Line	Chromosomal Location	Ectopic expression
pUAST	NA	2-6	II	None
		25-1	III	None
1-440 (full)	none	18-1	II	None
				None
		34-1	III	Yes
				Larval lethal
				None
Δ1-87	N-terminal	7-1	III	Yes
		7-2	II	Yes
		8-3	III	Yes
				Larval lethal
				None
Δ220-281	VID	4-7	II	None
		23-1	II	None
Δ282-344	VID	1-1	II	None
		2-6	III	None
Δ220-344	VID	1-4	III	None
		1-6	II	None
		2-6	II	None
88-159	N & C-terminal, linker, & VID	40-1	III	None
88-167	N & C-terminal, linker, & VID	8-1	III	None
88-167 + 345-440	N-terminal, linker, & VID	23-1	III	None
Δ88-123	DNA binding	2-2	III	None
		2-8	II	None
		3-1	II	None
Δ124-159	DNA binding	6-2	III	None
		13-7	III	None
Δ88-159 Not blunt	DNA binding	9-8	II	None
		9-10	III	None
Δ88-159 blunt	DNA binding	2-1	III	None
		38-1	II	None

Table 4.1b Summary of the ectopic assay continued

Construct	Domain(s) interrupted	Line	Chromosomal Locations	Ectopic expression
Δ1-200	DNA binding	5-4 10-1 11-2 15-1	II II II III	None None None None
88-167 + 220-344	N & C-terminal, & linker	19-1	III	None
Δ434-440	C-terminal	17-1 21-2	II II	None None
Δ416-440	C-terminal	9-2	III	None
Δ391-440	C-terminal	29-1	III	None
Δ365-440	C-terminal	8-1	III	None
Δ345-440	C-terminal	31-2	II	None
Δ345-415	C-terminal	15-1	III	None
Δ416-433	putative finger motif	30-1	II	None
Δ160-219 not blunt	linker	5-1 12-1 36-1	III II III	None None None
Δ160-219 blunt	linker	17-1 24-1 25-1	II II III	None None None
Δ168-219	linker	6-1 9-1	III III	Pupal lethal Larval lethal
Δ168-219R	linker	39-1 40-1	III II	Larval lethal Pupal lethal
137-219	N & C-terminal, DNA binding, & VID	16-1	II	None
88-219	N & C-terminal, & VID	12-1	III	None
137-344	N & C- terminal, & DNA binding	19-1	II	None
137-219 + 345 - 440	N-terminal, DNA binding, & VID	19-1	III	None

Blue designates assays performed at 18^0C, black at room temperature, and red indicates 29^0C. At 29^0C, both full length Sd ($^\#$34-1), and Δ1-87 ($^\#$7-1, $^\#$7-2, and $^\#$8-3) were lethal to the fly when expressed with *ptc*GAl4. Interestingly, constructs Δ168-219, and Δ168-219R were also lethal when expressed with the same driver.

Figure 4.1: Third instar wing discs showing ectopic expression of *sd* along the A/P boundary. Expression of all three independent lines of Sd Δ1-87 (#7-1, #7-2, and #8-3) induced ectopic expression of *sd*, as shown representatively in panel (a) with line #8-3. (b) Expression of full length Sd #34-1 can also induce ectopic *sd* expression, while line #18-1 does not (data not shown). The arrows indicate ectopic *sd* expression. (c) UAS-lacZ/*ptc*Gal4 staining to show the expression pattern produced by this driver and (d) shows a negative control pattern for ectopic expression produced with the empty injection vector pUAST line #2-6. The staining pattern shown here is also typical of the normal endogenous *sd* expression. When the ectopic expression assay is performed at 18^{0}C rather than at room temperature, the level of ectopic *sd* expression is virtually eliminated using either (e) Sd Δ1-87 #8-3 or (f) full length Sd #34-1.

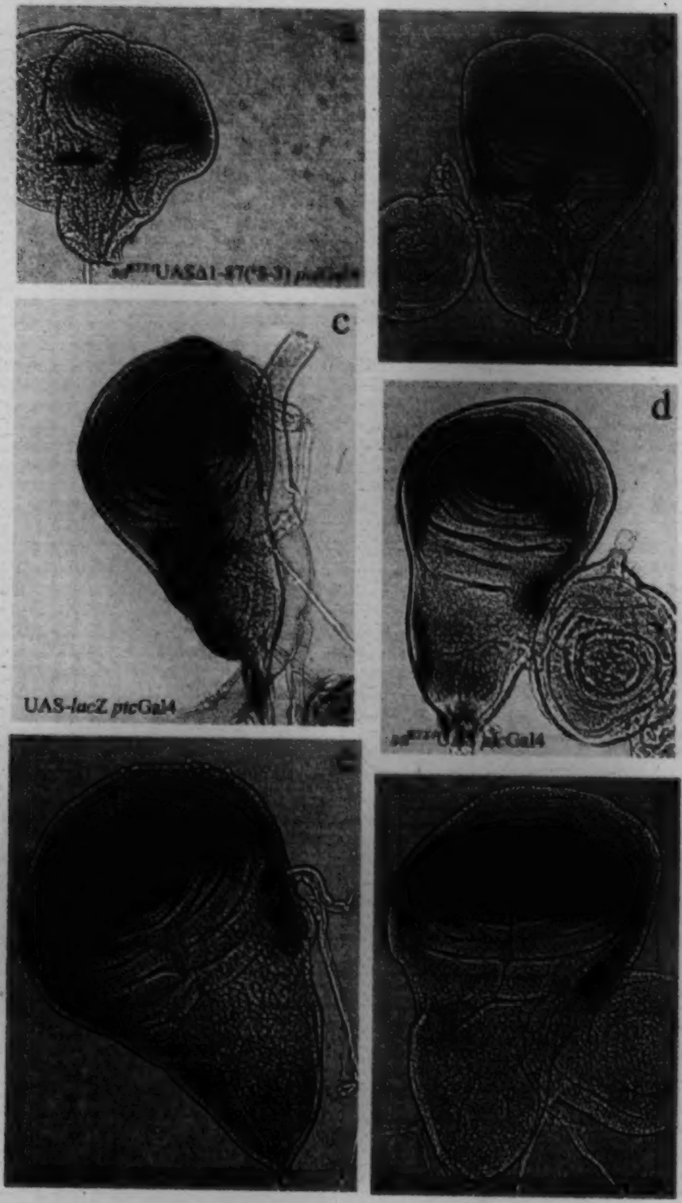
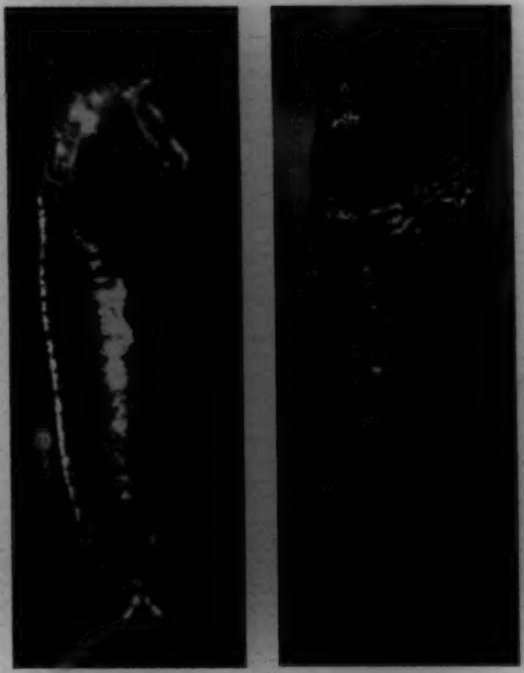

UASΔ1-87(C8-3)
c
UAS-lacZ ptcGal4
d
cGal4

Figure 4.2: Constructs Δ160-219 #6-1, and Δ168-219R #40-1 cause a pupal lethal phenotype when expressed with *ptc*Gal4. Panel (a) is a wildtype pupa approximately ten days after egg-laying and raised at room temperature. (b) Shows an otherwise wildtype pupa with construct Δ160-219 #6-1 expressed with *ptc*Gal4 approximately 14 days after egg-laying and raised at room temperature. Note the slower developmental rate for these organisms to develop into a pupa. The phenotype for Δ168-219R #40-1 is as Δ160-219 #6-1 (data not shown).



4.3 Discussion:

4.3-1 Results from the ectopic expression assay confirm findings from the wing rescue assay

Transgenic flies expressing full length Sd line #34-1 are able to induce ectopic endogenous *sd* expression while full length Sd line #18-1 cannot. It is unclear why this is the case. One possibility is that the expression level of full length Sd #18-1 is insufficient to induce ectopic *sd* expression but can rescue the *sd* tester mutants in the wing rescue assay (chapter 3). With respect to full length Sd #34-1, its expression level is sufficient to drive ectopic expression of endogenous *sd* and cause a dominant negative phenotype in the wing rescue assay. Expressions of all three independent lines of Δ1-87 have the ability to induce ectopic *sd* expression. In addition, at 18^0C, the ectopic *sd* expression induced by expression of full length Sd #34-1 and all three independent lines of Δ1-87 diminished drastically, suggesting that the level of Sd affects its ability to induce endogenous *sd* expression. Perhaps the expression levels of all three independent lines of Δ1-87 are at the "appropriate level" to induce ectopic *sd* expression. This also illustrates that the N-terminal 87 amino acids of Sd are dispensable for an ability to activate endogenous *sd* expression. This agrees with the results from the wing rescue assay in that the entire Sd protein excluding the N-terminal 87 amino acids is required for its function in wing development.

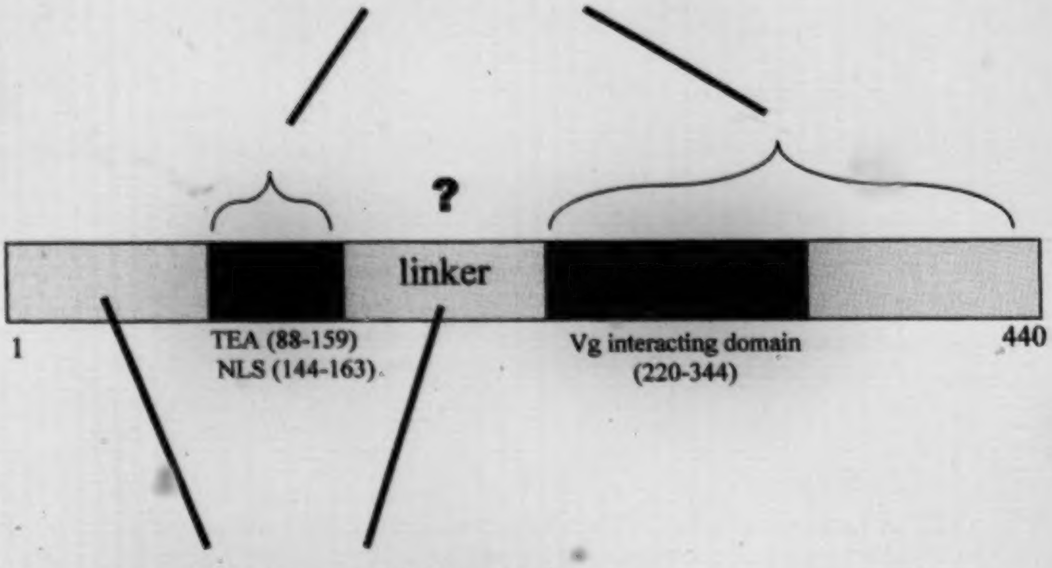
Expressions of all constructs that delete part or the entire C-terminal domain of Sd did not cause ectopic expression of *sd*. This serves as further evidence to support the notion that the entire C-terminal portion of Sd (from 345 to 440 of the protein) is required for a stable Sd-Vg complex. When the C-terminal domain is interrupted, the association of Vg with Sd may not be stable, which can compromise the ability of the Sd-Vg complex to activate *sd* expression. Furthermore, the remaining constructs shown in Table 4.1a and b are also unable to induce ectopic endogenous *sd*. This indicates that Sd has to form a stable interaction with Vg (by retaining the VID, and the C-terminal domain) as well as being able to bind its DNA target (thus the TEA DNA binding domain is also required) in order to activate endogenous *sd* expression. It is uncertain whether the linker domain is also required for ectopic expression of *sd*. The lack of ectopic expression in transgenics expressing construct Δ160-219 could be attributed to having part of the putative NLS interrupted, rendering these aberrant Sd proteins unable to enter the nucleus to drive ectopic endogenous *sd* expression. Unfortunately, when only the linker domain is deleted and the entire putative NLS is intact (construct Δ168-219 #6-1), this caused the surviving larvae to be very unhealthy, making it difficult to assay for ectopic expression of *sd*.

When transgenic lines harboring constructs Δ168-219, and Δ168-219R are expressed via the *ptc*Gal4 driver at room temperature, they cause a lethal phenotype as previously mentioned. Coincidentally, when transgenic lines harboring full length Sd #34-1 and Δ1-87 (#7-1, #7-2, and #8-3) are expressed at 29^{0}C, this also causes all the larvae to die. This hints to the involvement of Sd in the biological development of the fly, as also suggested by the presence of *sd* lethal alleles.

The expression of full length Sd, Δ1-87, Δ168-219, and Δ168-219R in the temporal and spatial pattern of the *ptc* promoter as described in Materials and Methods (section 2.17) might have contributed to the larval lethal phenotype. The pupal lethal phenotype might be explained by a lower expression level from Δ168-219 #6-1, and Δ168-219R #40-1, such that it enabled the organism to survive to the pupal stage of development. Unfortunately, the mechanism by which these constructs can cause a lethal phenotype is not known. However, it is interesting to note that expression of Sd Δ160-219 with part of the putative NLS and the entire linker domain deleted did not cause a lethal phenotype. It is possible that when the putative NLS is interrupted, this compromises the ability of the mutant Sd to enter the nucleus, therefore, rendering it unable to cause a lethal phenotype. Nevertheless, these results suggest that Sd deleted for the N-terminal 87 amino acids and the linker domain can behave in a similar fashion as the full length protein and cause lethality to the organism when mis-expressed with *ptc*Gal4. Furthermore, this indicates that the N-terminal 87 amino acids and the linker domain are dispensable for the lethal effect of Sd. This suggests that these two domains may not be required for Sd's role in vitality of the flies. This coincides with the finding that none of the presently characterized *sd* lethal alleles map to these two domains of Sd (Srivastava et al., 2004). A future experiment to test this theory would be to determine the ability of full length Sd, Δ1-87, Δ168-219, and Δ168-219R to rescue *sd* lethal alleles. If the N-terminal 87 amino acids and the linker domain are not required for the vital biological functions of Sd, these constructs may be able to rescue *sd* lethal mutants. A summary of the domains of Sd required for ectopic endogenous *sd* expression and viability in *Drosophila* is shown in Figure 4.3.

Figure 4.3: Functional domains of Sd with respect to ectopic expression of endogenous *sd*, and its vital functions. The red lines indicate regions of Sd that when deleted still retained the ability to cause lethality to the organism, as when full length *sd* is expressed with the *ptc*Gal4 driver. The regions of Sd required for ectopic endogenous *sd* expression are indicated by the blue lines and brackets. It is uncertain whether the linker domain is required for ec[illegible]pic expression of *sd* as indicated by the "?" symbol.

Regions important for ectopic expression of endogenous *sd*


?
linker
1
TEA (88-159)
NLS (144-163)
Vg interacting domain
(220-344)
440

When these regions were deleted or replaced, the truncated protein still behaved as full length Sd and was capable of causing lethality.

Chapter 5: Functional dissection of the role of Sd in *Drosophila* eye development

5.1 Introduction

There are several pieces of evidence to suggest that Sd has a role in *Drosophila* eye development. A *sd* reporter gene is expressed behind the MF as it migrates across the developing eye disc during the third larval instar (Campbell et al., 1992). The original X-ray induced *sd* viable allele sd^1 (Gruneberg, 1929) in addition to having a wing phenotype, manifests a slight roughening of the adult compound eye suggesting a possible role of Sd in eye development (Lindsley and Zimm, 1990). A missense mutation in the C-terminal end of the human homolog of *sd*, TEF-1, is responsible for an eye disease in humans called Sveinsson's chorioretinal atrophy (SCRA) (Fossdal et al., 2004). Finally, a Yeast two hybrid screen has identified the *ninaA* (*neither inactivation nor afterpotential A*) gene product as a possible interacting protein with Sd. NinaA is an integral membrane protein required for the proper functions of rhodopsin 1 (visual pigment) in *Drosophila* eyes (Stamnes et al., 1991). Coincidentally, *sd* reporter expression is found in adult photoreceptor cells (Campbell et al., 1992) where rhodopsin is synthesized (Stamnes et al., 1991). Taken together, these data strongly suggest a functional role for Sd in eye development.

To gain some insight into what role Sd may play in development of the eye, all the *sd* deletion constructs generated were assayed for their respective ability to cause an eye phenotype when over-expressed in an otherwise wildtype background. In addition to understanding which regions of Sd may be important for eye morphogenesis, data from this assay could also shed light into the possible role of TEF-1 in human eye development.

5.2 Results:

5.2-1 Expression of the following Sd constructs did not cause an eye phenotype

To study which region(s) of Sd may affect proper eye development, an over-expression assay in the compound eye was carried out as described in Materials and Methods section 2.19. Table 5.1 summarizes the results of the *sd* deletion constructs assayed for their respective ability to cause an eye phenotype. Results herein are based on the over-expression assay performed at room temperature unless otherwise indicated. As a negative control, over-expression of the injection vector pUAST did not cause an eye phenotype (data not shown). Over-expression of all independent lines of full length Sd, Δ1-87, constructs that interrupt the C-terminal domain (Δ345-440, Δ365-440, Δ391-440, Δ416-440, Δ434-440, Δ345-415, Δ416-433), and constructs that interrupt the VID (Δ220-281, Δ282-344, Δ220-344) had no effect in the over-expression assay. Furthermore, constructs retaining the amino acids encoding capacity for residues 88-159 , 88-167, 137-219, 88-219, 137-344, 137-219+345-440, 88-167+345-440, and 88-167+220-344 also did not cause an eye phenotype. See Figure 2.1 for an illustration of these *sd* deletion constructs.

Table 5.1a Summary of the eye over-expression assay

Constructs	Domain(s) interrupted	Line	Chromosomal Location	Ectopic expression
pUAST	NA	2-6	II	None
		25-1	III	None
1-440 (full)	none	18-1	II	None
		34-1	III	None
Δ1-87	N-terminal	7-1	III	None
		7-2	II	None
		8-3	III	None
Δ220-281	VID	4-7	II	None
		23-1	II	None
Δ282-344	VID	1-1	II	None
		2-6	III	None
Δ220-344	VID	1-4	III	None
		1-6	II	None
		2-6	II	None
88-159	N & C-terminal, linker, & VID	22-1	III	None
		40-1	III	None
88-167	N & C-terminal, linker, & VID	8-1	III	None
		25-1	III	None
88-167 + 345-440	N-terminal, linker, & VID	23-1	III	None
Δ88-123	DNA binding	2-2	III	None
		2-8	II	None
		3-1	II	None
Δ124-159	DNA binding	6-2	III	Present (24/55 or 44%)
		13-7	III	Present (42/110 or 38%)
				Present (73/90 or 81%)
				Present (11/58 or 19%)
Δ88-159 Not blunt	DNA binding	9-8	II	*Present (10/61 or 16%)
		9-10	III	*Present (6/81 or 7%)
Δ88-159 blunt	DNA binding	7-1	II	*Present (1/54 or 2%)
		38-1	II	*Present (19/62 or 31%)
Δ1-200	DNA binding	5-4	II	Present (8/52 or 15%)
		10-1	II	Present (10/50 or 20%)
				Present (61/85 or 72%)
				Present (9/53 or 17%)
		11-2	II	Present (8/66 or 12%)

Table 5.1b Summary of the eye over-expression assay continued

Construct	Domain(s) interrupted	Line	Chromosomal Locations	Ectopic expression
88-167 + 220-344	N & C-terminal, & linker	19-1	III	None
		28-1	III	None
Δ434-440	C-terminal	17-1	II	None
		21-2	II	None
Δ416-440	C-terminal	9-2	III	None
		11-1	III	None
Δ391-440	C-terminal	9-1	III	None
		29-1	III	None
Δ365-440	C-terminal	8-1	III	None
		24-1	II	None
Δ345-440	C-terminal	31-2	II	None
		31-4	III	None
Δ345-415	C-terminal	15-1	III	None
		21-1	II	None
Δ416-433	putative finger motif	23-1	III	None
		30-1	II	None
Δ160-219 not blunt	linker	6-1	II	*Present (5/52 or 10%)
		12-1	II	*Present (5/82 or 6%)
Δ160-219 blunt	linker	24-1	II	*Present (3/70 or 4%)
		25-1	III	*Present (4/63 or 6%)
Δ168-219	linker	6-1	III	*Present (24/47 or 51%)
		9-1	III	‡Present (11/13 or 85%) -pupal lethal
		9-2	III	*Present (33/48 or 69%)
Δ168-219R	linker	39-1	III	‡Present (8/8 or 100%) -pupal lethal
		40-1	II	‡Present (29/51 or 57%) -pupal lethal
137-219	N & C-terminal, DNA binding, & VID	16-1	II	None
88-219	N & C-terminal, & VID	8-1	II	None
		12-1	III	None
137-344	N & C- terminal, & DNA binding	19-1	II	None
137-219 + 345 - 440	N-terminal, DNA binding, & VID	19-1	III	None
		24-1	II	None

Blue designates assays performed at 18^{0}C, black at room temperature, and red indicates 29^{0}C. *indicates eye phenotypes that are less severe than transgenic flies over-expressing

Δ1-200 and Δ124-159. Often the eye protrusion affects only one eye. ‡indicates other phenotypes observed in addition to the asymmetric eye outgrowth phenotype. These phenotypes include a combination of the following features: ectopic and mislocalization of the ocelli, duplication of the antenna, drastic reduction of eye size, deformation of the head, and pupal lethal phenotype.

5.2-2 Expression of the following Sd constructs caused an eye phenotype

The eye phenotypes described are typical of those caused by expression of the respective Sd construct under discussion. However, it is worthwhile to note that there are slight variations in severity among the eye phenotypes associated with each transgenic line.

Expression of constructs interrupted in the TEA DNA binding domain cause a surprising eye phenotype. Transgenic lines harboring construct Δ1-200 (#5-4, #10-1, and #11-2) which has the N-terminal and the entire TEA DNA binding domain deleted caused 15% (8/52), 20% (10/50), and 12% (8/66) of the flies to manifest an unusual outgrowth of the eyes, respectively (Figure 5.1). Expression of construct Δ124-159, deleted for only the second half of the TEA DNA binding domain, exhibits the highest penetrance of this eye phenotype, with expression of lines #6-2, and #13-7 causing 44% (24/55), and 38% (42/110) of the flies to show the outgrowth phenotype, respectively (Figure 5.2c & d). In flies with the outgrowth phenotype, the ommatidia and eye bristles are also disorganized in comparison to their systematic arrangement in wildtype eyes (Figure 5.2a & b). Fusions of the ommatidia and eye bristles are also apparent. Flies expressing construct Δ88-159nb (#9-8, and #9-10) have 16% (10/61), and 7% (6/81) of the flies with an eye phenotype, respectively (Figure 5.3a), whereas flies expressing construct Δ88-159b (#7-1, and #38-1) have only 2% (1/54), and 31% (19/62) of the flies with an eye phenotype, respectively (Figure 5.3b). Eye phenotypes associated with Δ88-159 nb/b are weaker than the phenotypes associated with Δ1-200 and Δ124-159. The outgrowths of the eyes are less protruding and often affect one instead of both compound eyes. Although

Figure 5.1: Over-expression of construct Δ1-200 with *ey*Gal4 results in an eye outgrowth phenotype. (a) Over-expression of Δ1-200 (#10-1) via *ey*Gal4 in a wildtype eye background. Notice the protrusion of both compound eyes. (b) Magnification of the right compound eye and (c) the left compound eye to 600X from the fly in panel a. Note the disorganization of the ommatidia and eye bristles in comparison to wildtype eyes in Figure 5.2 a & b. (d) Magnification of the left compound eye of the same fly to 1000X, the red arrows point to fusion of the ommatidia and yellow arrows indicate the fusion of the eye bristles. Results for the other independent lines of Δ1-200 are similar and are not shown. Abbreviations: RE; right compound eye, LE; left compound eye and will be used for all figures to follow in this chapter.

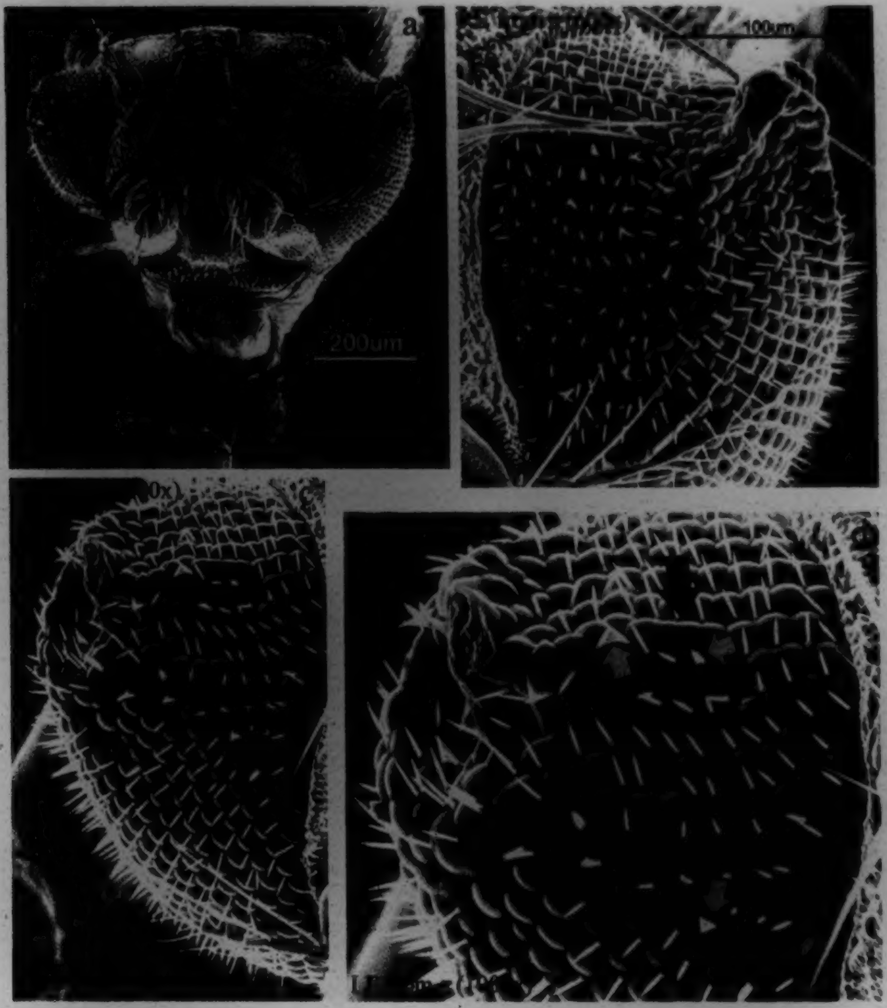

a
200um
100um
0x)
c
d

Figure 5.2: Over-expression of construct Δ124-159 with *ey*Gal4 results in an eye outgrowth phenotype similar to flies over-expressing Δ1-200. (a) An Oregon R. *Drosophila* head and (b) the left compound eye of this fly magnified to 600X. Notice the round and symmetrical compound eyes, the honeycomb like arrangement of ommatidia, and the eye bristles arranged in an organized pattern. Panel (c) is an otherwise wildtype fly over-expressing construct Δ125-159 (#6-2) and (d) magnification of the right compound eye of this fly to 600X. Notice the protrusion of the eye in (c) and the disorganization of the ommatidia and eye bristles in (d). The red arrows indicate fusion of the ommatidia and yellow arrows illustrate fusion of the eye bristles. Results for over-expression of construct Δ125-159 (#13-7) are similar (data are not shown).

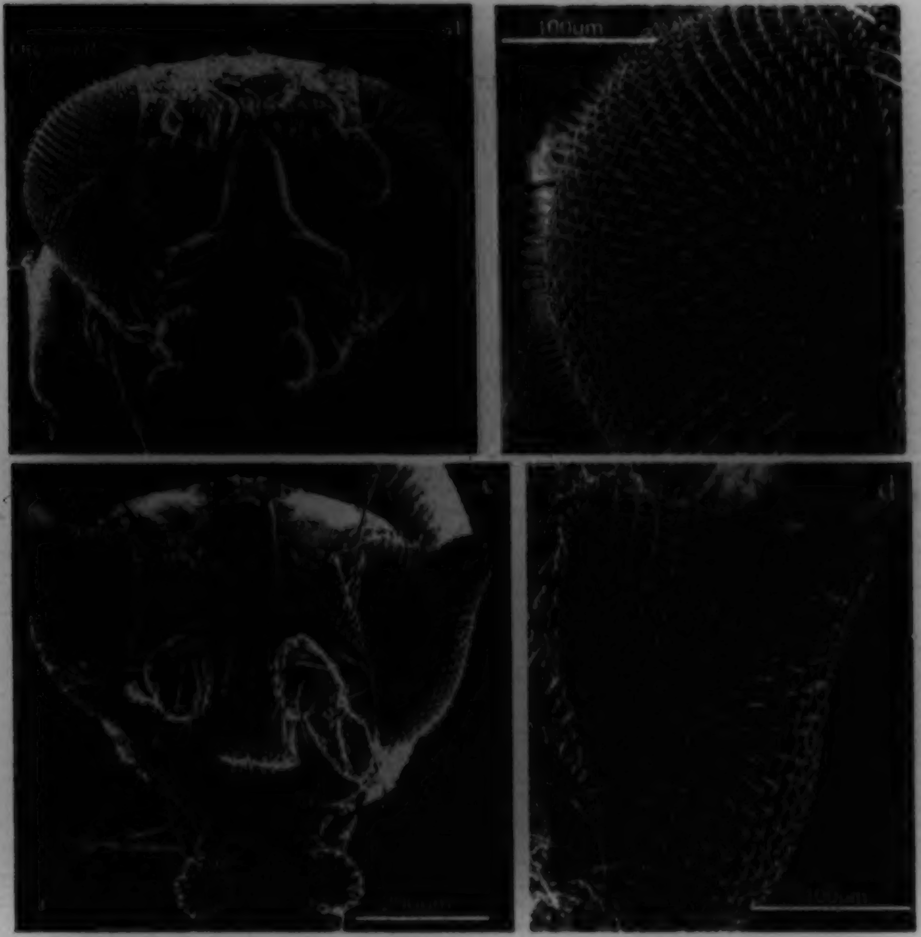
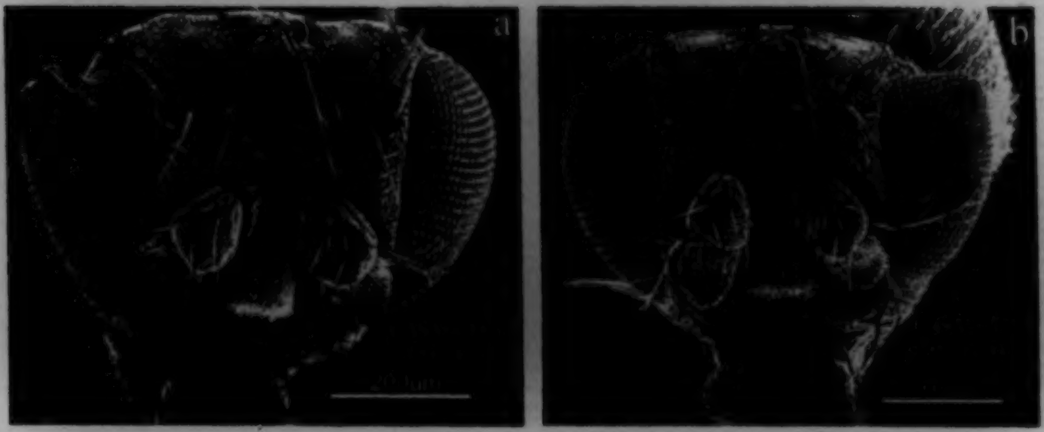

100um
d

Figure 5.3: Transgenic flies over-expressing construct Δ88-159 manifest a weaker eye phenotype than those over-expressing construct Δ1-200 and Δ125-159. Panel (a) and (b) show over-expression of Δ88-159nb ($^{\#}$9-8) and Δ88-159b ($^{\#}$38-1), respectively, using *ey*Gal4. The eye outgrowth phenotype associated with these constructs is weaker and the protrusion often affects one instead of both compound eyes. Fusions of ommatidia and eye bristles also occur less often. The *Spe*I linker does not appear to have an effect in this over-expression assay. The eye phenotypes associated with other independent lines of Δ88-159 b/nb are similar (date are not shown).


a
b
200 µm

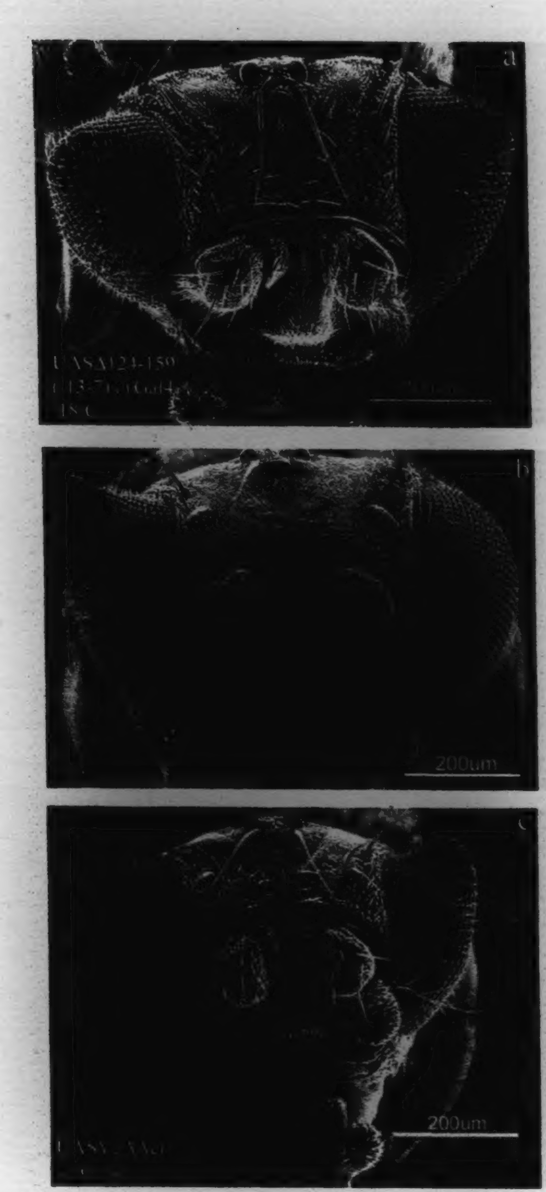
disorganization of the ommatidia and eye bristles are still present, fusions of ommatidia and eye bristles occurred less frequently. Therefore, it appears that over-expression of Sd interrupted in the TEA DNA binding domain can cause an eye outgrowth phenotype.

The results of this assay are also affected by temperature. At 29^0C, transgenics of Δ1-200 ($^{\#}$10-1) and Δ124-159 ($^{\#}$13-7) exhibited an eye phenotype in 72% (61/85) and 81% (73/90) of the flies compared to 20% and 38% at room temperature, respectively. At 18^0C, transgenics of construct Δ1-200 ($^{\#}$10-1) and Δ124-159 ($^{\#}$13-7) caused an eye phenotype in only 17% (9/53), and 19% (11/58) of the flies, respectively. In addition, the eye phenotype is less severe at 18^0C compared to room temperature and 29^0C (Figure 5.4a & b). The outgrowths of the eyes are less protruding and sometimes only one eye is affected.

A possible explanation for the eye phenotype is that Sd may interact with an unknown factor (X) in the eye to promote proper eye morphogenesis. By over-expressing constructs interrupted in the TEA DNA binding domain (assumed to lack the ability to bind DNA targets in the eye), the proteins produced from these constructs could out-compete endogenous Sd for X, resulting in an eye phenotype. To test this theory, Vg containing only the Sd interacting domain (VgΔAct) (Ajay Srivastava, unpublished work) was expressed with *ey*Gal4. Presumably, this construct would bind and titrate endogenous Sd from X. Interestingly, expression of this construct causes an eye outgrowth phenotype similar to those over-expressing Δ1-200 and Δ124-159 (Figure 5.4c).

Expression of constructs interrupted in the linker domain including Δ160-219 nb/b, Δ168-219, and Δ168-219R also cause an eye phenotype. Transgenics of constructs

Figure 5.4: The eye over-expression assay is affected by temperature. Transgenic flies over-expressing constructs Δ124-159 (#13-7), and Δ1-200 (#10-1) at 18^{0}C with *ey*Gal4 in (a) and (b) respectively. The eye outgrowth is less severe and the proportion of flies exhibiting the eye phenotype is lower compared to results at room temperature. Panel (c) shows an otherwise wildtype fly over-expressing construct VgΔAct with *ey*Gal4. Notice the eye phenotype is very similar to transgenic flies over-expressing construct Δ1-200 and Δ124-159.


a
UASA124-159
b
200um
c
200um

Δ160-219nb (#6-1), and (#12-1) caused 10% (5/52), and 6% (5/82) of the flies to manifest an eye phenotype (Figure 5.5a). Expression of Δ160-219b (#24-1, and #25-1) caused the eye phenotype in 4% (3/70), and 6% (4/63) of the organisms, respectively (Figure 5.5b). The outgrowths associated with these constructs were less severe compared to flies over-expressing Δ1-200 and Δ124-159. In addition, the outgrowth also often affects one instead of both compound eyes. Surprisingly, expression of constructs Δ168-219, and Δ168-219R also cause malformations of the *Drosophila* head, in addition to causing an eye phenotype. Again, the eye outgrowth often affects one instead of both eyes and is less protruding than in flies over-expressing Δ1-200 and Δ124-159. Within the transgenic flies over-expressing construct Δ 168-219, the phenotypes associated with Δ 168-219 line #9-1 were much more severe than Δ 168-219 lines #6-1 and #9-2. Flies expressing construct Δ 168-219 (#6-1 and #9-2) caused 51% (24/47), and 69% (33/48) of the flies to manifest an eye phenotype (Figure 5.5c). However, with construct Δ168-219 (#9-1) most of the larvae died at the pupal stage. Among the survivors, 85% (11/13) exhibited a combination of the following features: asymmetry between the compound eyes, ectopic expression and mislocalization of the ocelli, duplication of the antenna, and overall deformation of the head (Figure 5.6). Both transgenic lines of construct Δ168-219R (#39-1 and #40-1) exhibit a pupal lethal phenotype. However, the effects associated with line #39-1 were more severe. For example, expression of construct Δ168-219R #39-1, and #40-1 caused 100% (8/8) and 57% (29/51) of the surviving flies to exhibit phenotypes such as those described for Δ168-219 (#9-1), except there is a severe asymmetry between the compound eyes within one organism. In some cases the eyes are absent or drastically reduced in size (Figure 5.7).

Figure 5.5: Expression of *sd* deletion constructs interrupted in the linker domain also cause an eye phenotype. Transgenic flies over-expressing construct Δ160-219nb (#6-1), and Δ160-219b (#25-1) with *ey*Gal4 in (a) and (b), respectively. Again, the eye phenotype associated with these constructs is less severe compared to transgenic flies over-expressing Δ1-200 and Δ124-159. The *Spe*I sequence appears to have no effect in the eye over-expression assay. Results for other independent lines of Δ160-219 nb/b are similar and are not shown. Panel (c) is a transgenic fly over-expressing construct Δ168-219 (#6-1). The eye outgrowth often affects one instead of both eyes. Results for Δ168-219 (#9-2) are similar (data are not shown).

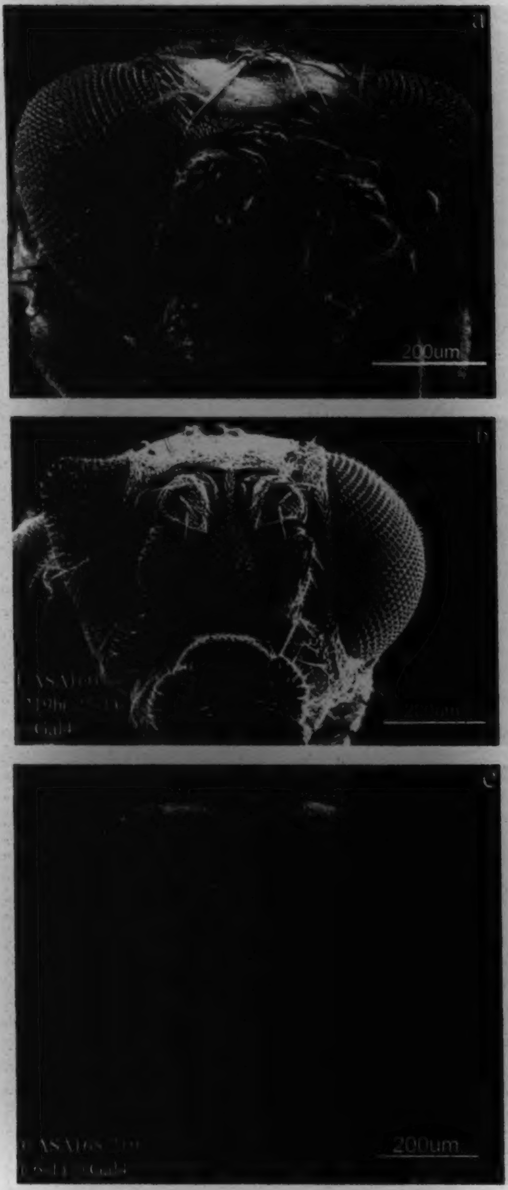
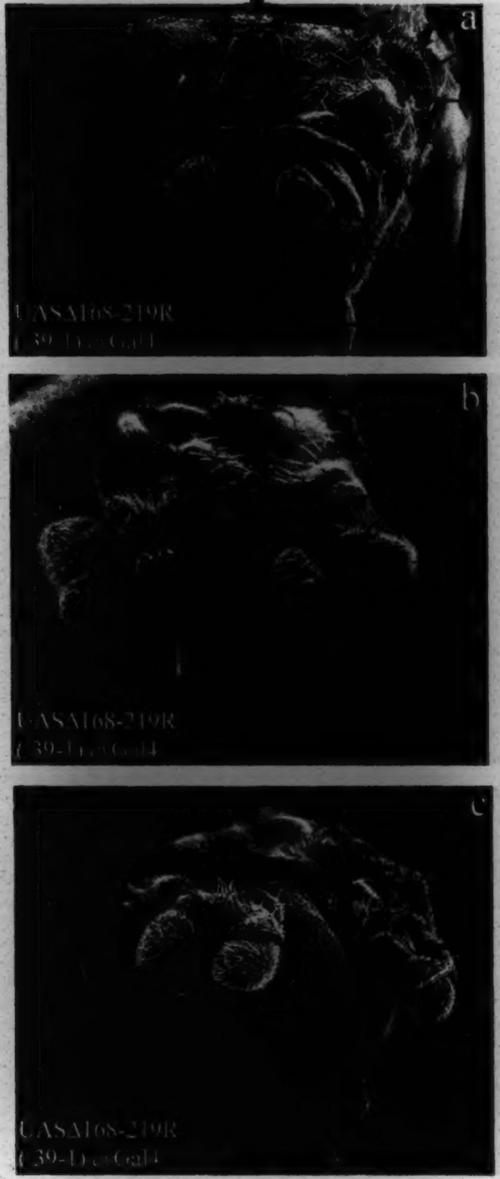

a
200um
b
200um
c
200um

Figure 5.6: Over-expression of construct Δ168-219 causes other head deformations and a pupal lethal phenotype in addition to the eye phenotype. Panel (a) and (b) show two different surviving flies over-expressing construct Δ168-219 (#9-1). Notice in (a) the asymmetry between the two eyes, and deformation of the head. Ectopic and mislocalized ocelli are indicated by the blue arrows. The green arrow indicates the wildtype location of the ocelli. The fly in (b) has duplication of the antenna (orange arrow) in addition to exhibiting asymmetry between the two eyes.

a
UASΔ168-219
C9-D;eyGal4
b
UASΔ168-219
C9-D;eyGal4

Figure 5.7: Transgenic flies over-expressing construct Δ168-219R show similar phenotypes to transgenic flies over-expressing construct Δ168-219 (#9-1). Surviving transgenic flies over-expressing Δ168-219R (#39-1) and (#40-1) have a severe asymmetry between the two compound eyes. Panel (a), (b), and (c) are survivors over-expressing construct Δ168-219R #39-1. In (a) the blue arrow shows mislocalization and ectopic expression of the ocelli, the green arrow indicates the wildtype location of the three ocelli and the brown arrow illustrates the drastic reduction in the size of one compound eye. In (b) one of the eyes is absent and appears to be replaced by a single ocellus (indicated by the blue arrow). The remaining eye is greatly reduced in size (indicated by the brown arrow). In (c) both of the compound eyes are absent and there is duplication of the antenna as designated by the orange arrow.


a
UASΔ168-219R
C39-1D::Gal4
b
UASΔ168-219R
C39-1D::Gal4
c
UASΔ168-219R
C39-1D::Gal4

5.3 Discussion:

5.3-1 Sd may have a role in cell growth regulation in the eye

The eye outgrowth phenotype may be due to an over-proliferation of the eye imaginal disc cells where the *ey*Gal4 driver is active. For the temporal and spatial expression pattern of the *ey*Gal4 driver, see Materials and Methods (section 2.19). During the third instar larval stage, differentiated cells posterior to the MF assemble into approximately 750 ommatidia, leaving behind about 2000 cells that are eliminated by apoptosis about 36 hours after puparium formation (Huang et al., 2005; Wolff and Ready, 1993). Yorki (Yki) is the *Drosophila* homolog of YAP (yes-associated protein), a co-activator in mammalian cells (Strano et al., 2002; Vassilev et al., 2001; Yagi et al., 1999) and is known to be involved in regulation of cell proliferation and apoptosis in the Hippo Signaling Pathway (Huang et al., 2005). Increased cell proliferation and inhibition of normal developmental cell death were found in *Drosophila* tissues over-expressing *yki* (Huang et al., 2005). The product of *yki* is also required for tissue growth (Huang et al., 2005). YAP is known to bind TEAD-1, the mouse homolog of Sd. Furthermore, TEF-1, the human homolog of TEAD-1, appears to have a role in human eye development (Fossdal et al., 2004). Therefore, there appears to be a link between the function of Sd and cell growth regulation in the eye. Coincidentally, Sd had been found to be correlated with cell survival in the wing and leg imaginal disc (Garg et al., 2006). In addition, Sd is also known to be involved in cell growth regulation in the wing tissue (Delanoue et al., 2004; Liu et al., 2000). The disorganization and fusion of the ommatidia and bristles could be an outcome of the over proliferation of the eye or it could involve other unknown function(s) of Sd in eye development.

5.3-2 Sd may interact with an unknown factor in the eye for proper development of this tissue

The data so far suggest that Sd may interact with an unidentified factor in the eye for proper development of this tissue. Since the identity of this factor in the eye is not known, the theoretical Sd co-factor in the eye will be referred to as X hereafter. Presumably, the eye phenotype results when X is titrated from endogenous Sd. This notion considers the possibility that X is the rate limiting factor in the formation of the Sd-X complex. To support the theory that this Sd complex has a role in eye development, over-expression of construct VgΔAct caused a similar eye phenotype. It is possible that ectopically expressed VgΔAct can bind and titrate endogenous Sd from X. Without Sd, X is unable to serve its role in eye development, thus resulting in the outgrowth phenotype.

When the over-expression assay was performed at a higher temperature (29^0C), more flies exhibited the eye phenotype when Δ1-200, and Δ124-159 were over-expressed. Conversely, when these constructs were expressed at a lower temperature (18^0C), there were fewer flies with the eye phenotype and the outgrowth became less severe. Since the Gal4 driver is more active at a higher temperature (Brand et al., 1994, Kumar and Moses 2001, Speicher et al., 1994), these results suggest that when the transgene is expressed at a higher level, it is able to titrate X from endogenous Sd more efficiently, causing a more severe phenotype with a higher proportion of flies exhibiting the eye phenotype. Conversely at a lower temperature, less transgene product is produced to titrate X from endogenous Sd, resulting in a less severe phenotype and a lower proportion of organisms with the eye phenotype.

Expression of construct Δ88-159, which deletes the entire TEA DNA binding domain, causes a weaker eye protrusion phenotype, often affecting only one compound eye. In addition, expression of construct Δ88-123, which deletes the first half of the TEA DNA binding domain, does not cause an eye phenotype. This might be due to a lower level of expression from these constructs and hence a weaker ability to out-compete X from endogenous Sd. It is possible that there is a threshold level of expression of the *sd* transgene that is necessary to bind X and out-compete the binding of endogenous Sd to cause an eye phenotype. If the level of expression is below the threshold, there is no eye phenotype. However, if the expression level is above the threshold this results in an eye phenotype. Alternatively, deleting the N-terminal 200 amino acids or residues 124-159 from Sd may cause a conformational change in these proteins such that they have an enhanced ability to bind X. This allows Δ1-200, and Δ124-159 to bind X more effectively and out-compete endogenous Sd, causing a more extreme eye phenotype.

5.3-3 Why over-expression of some Sd constructs had no effect in eye development

Over-expression of full length Sd and Δ1-87 have no effect in the eye. Perhaps in the eye tissue, formation of the Sd-X complex is not dependent on the relative concentration of the two proteins. The condition for formation of Sd-X might simply require the presence of the two molecules to interact. Therefore, when Sd and Δ1-87 are over-expressed, the Sd-X complex is still formed to serve its wildtype function in eye development. Expression of Sd constructs interrupted in the VID or the C-terminal domain have no effect in the over-expression assay either. This is consistent with the notion that the interaction domain between Sd and X may involve the entire VID and C-terminal domain of Sd. As mentioned previously, the human homolog of Sd, TEF-1, with the missense mutation (Y421H) known to cause SCRA is located within the C-terminal domain (Fossdal et al., 2004), suggesting that this region is important for its function in the eye. Further C-terminal truncations of Δ1-200 can be made to determine the minimal region of the C-terminal domain required to cause an eye phenotype. This may narrow down the minimal region required for X and Sd to interact in the eye tissue.

Expression of *sd* constructs retaining only the TEA DNA binding domain (87-159 & 87-167) also did not cause an eye phenotype. This suggests that the TEA DNA binding domain alone is unable to interact with target sites, and likely other regions of Sd are required for binding. If the TEA DNA binding domain alone was able to bind the same target sites as Sd-X, an eye phenotype would be expected. Finally, the following constructs tested (137-219, 88-219, 137-344, 137-219+345-440, 88-167+345-440, 88-167+220-344) also did not cause an eye phenotype. None of these constructs contain an entire VID and C-terminal domain of Sd. According to the idea that the entire VID and C-terminal region are required for interaction with X, then none of these constructs would be expected to cause an eye phenotype.

5.3-4 The linker domain of Sd serves an important yet unknown role in eye and head development

Expression of constructs interrupted in the linker domain are able to cause an eye phenotype. This suggests that an intact linker region is not required for Sd to interact with X. Consequently, these truncated proteins are likely able to bind X, titrating it from endogenous Sd to cause an eye phenotype. The eye phenotype associated with these constructs is weaker and often affects only one eye, causing an asymmetric eye phenotype. The reason for the asymmetric eye phenotype is not known. However, it could reflect slight differences in activity of the *ey*Gal4 driver between the two eye discs. It is possible that one eye disc may produce a threshold level of the *sd* transgene product to cause an eye phenotype while the level in the other eye disc is just below the threshold and does not affect the eye.

Surprisingly when Δ168-219 (#9-1) and Δ168-219R (#39-1, and #40-1) were over-expressed in an *ey*Gal4 pattern, there were other head malformations and lethal effects in addition to the asymmetric eye phenotype. The deformations found in the head might be why a large proportion of the flies do not survive through the pupal stage. However, the mechanism of how over-expression of these constructs can cause a lethal phenotype remains unclear.

Why the *sd* gene interrupted in the linker domain would cause any phenotype is also a mystery. Furthermore, Δ168-219, and Δ168-219R resulted in some common eye and head phenotypes. Even though it has been previously suggested that the linker domain is not required to interact with X, it is still possible that the linker domain of *sd* serves another unknown yet important function in eye development. If the linker domain is simply serving as a spacer between the TEA DNA binding domain and VID, Δ168-219R should behave as full length Sd in the over-expression assay and this is not what is observed. Moreover, the results from expressing construct Δ168-219 (retaining the putative NLS) serve as evidence to indicate that the eye phenotype associated with the Δ160-219 protein (interrupting the putative NLS) is not simply due to interruption of the putative NLS. Even when this region is retained, as in Δ168-219, this still caused both eye and head phenotypes. It is also very intriguing that Δ160-219 caused an eye phenotype whereas Δ168-219 resulted in other head phenotypes in addition to the eye phenotype. Perhaps Δ160-219, with the putative NLS interrupted is less able to enter the nucleus in comparison to Δ168-219, rendering it less able to titrate X from endogenous Sd. Consequently, flies over-expressing Δ168-219 exhibit other head phenotypes because the product can enter the nucleus. Nevertheless, the possible function of the linker domain in eye development is not known.

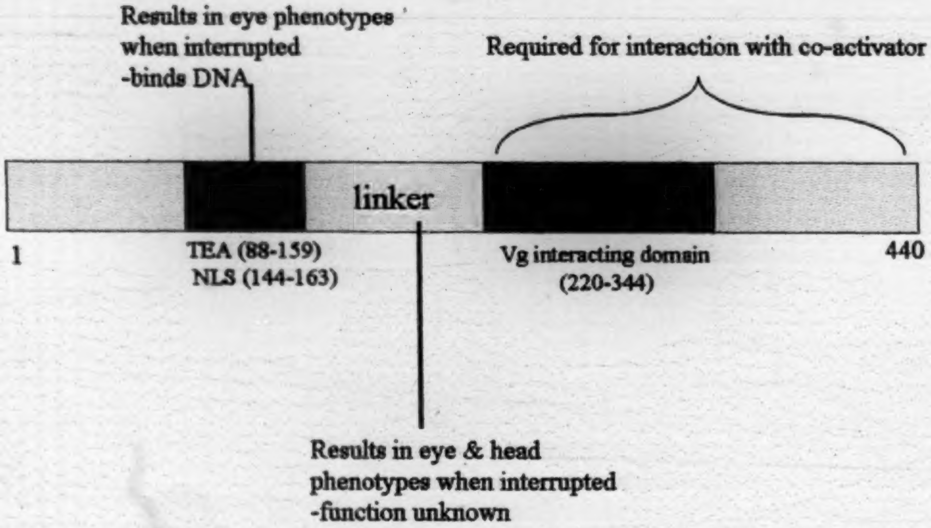
The deformation of the head might result from abnormal development of the eye-antennal discs when the larva becomes a pupa, at the point where the two eye-antennal discs evaginate and fuse to become the head structures. The connection between ectopic and mislocalization of the ocelli and over-expression of constructs Δ168-219 and Δ168-219R remains unclear. The ocelli develop from the eye portion of the eye-antennal disc (Figure 1.5). It was proposed by Punzo et al., (2002), that autoregulation of *sine oculis* (*so*) is required for ocellus development. Initially, *eyeless* (*ey*), and *twin of eyeless* (*toy*) mediate *so* expression in the early third instar throughout the eye disc, including the ocellar precursors (Figure 1.5) (Pauli et al., 2005; Punzo et al., 2002). After this first induction, So cooperates with the protein Eye Absent (Eya) to maintain its own expression in the ocellar region by a positive feedback loop (Pauli et al., 2005; Punzo et al., 2002). Although purely speculative, it is possible that expression of Δ168-219 and Δ168-219R may somehow interfere with this process to cause mislocalization and ectopic expression of the ocelli.

There is some evidence to support the involvement of Sd in antenna formation. The *sd*^{ETX4} allele discussed in previous chapters exhibits a taste defect (Anand et al., 1990). The olfactory sensory organ of *Drosophila* is located on the antenna and in a subset of cells in the sacculus of the antenna where *sd* reporter gene activity has been observed (Campbell et al., 1992). The *ey*Gal4 driver does express these constructs in the eye-antennal precursor cells and a strip in the third instar antennal disc (Hauck et al., 1999). Unfortunately, not much is known about the genetic hierarchy that governs antenna formation. What is known is that the antennae develop from the antennal portion of the eye-antennal disc and the Egfr and Notch (N) signaling pathways act in the specification of the eye and antenna (Kumar and Moses, 2001). Egfr is a transmembrane receptor kinase (RTK) that acts through the Ras pathway (Nilson and Schupbach, 1999; Schweitzer and Shilo, 1997) to promote an antennal fate while N signaling (Artavanis-Tsakonas et al., 1995) promotes an eye fate (Kumar and Moses, 2001). These pathways act upstream of the most currently assigned upstream eye specification gene *toy* (Kumar and Moses, 2001). Although not definitive, the data do suggest that over-expression of Sd Δ168-219 and Δ168-219R somehow impairs the normal development of the antenna. In short, however, how Δ168-219 and Δ168-219R can cause ectopic and mislocalization of the ocelli and duplication of the antenna is not understood.

5.3-5 Conclusions regarding the possible role of Sd in eye development

In summary, over-expression of *sd* constructs interrupted in the TEA DNA binding and linker domains resulted in an eye phenotype. It seems that Sd may have a role in cell growth regulation and other undefined functions in the eye. Sd may interact with an unknown eye factor to carry out these functions. It may also be involved in antennal development. Furthermore, the data obtained herein regarding its possible roles in eye and antennal development are still preliminary which makes any interpretation speculative at best. The next logical step would be to identify and characterize Sd's potential partner(s) in the eye-antennal disc, and this could serve as the first step in learning the role of Sd in these tissues. A summary of the domains important for its function in the eye-antennal disc is shown in Figure 5.8.

Figure 5.8: Summary of the functional domains of Sd in eye development. The blue lines point to regions that when interrupted caused an eye and/or head phenotypes. The region that may be required for Sd to interact with a potential co-activator in the eye is indicated by a bracket.


Results in eye phenotypes when interrupted
-binds DNA
Required for interaction with co-activator
linker
1
TEA (88-159)
NLS (144-163)
Vg interacting domain (220-344)
440
Results in eye & head phenotypes when interrupted
-function unknown

Chapter 6: Conclusions and future directions

6.1 How the Sd-Vg complex functions in wing development and the possible role of Sd in other tissues

The work presented strengthens some theories as to how Sd-Vg functions in wing development. First, the relative concentration of Sd and Vg are believed to be fundamentally important in the regulation of wing development (Halder et al., 1998b; Simmonds et al., 1998). In the wing rescue assay, over-expressing full length Sd caused malformation of the wing, but simultaneous over-expression of Vg alleviated this effect. Second, the *in vivo* data presented herein support the notion that Vg concentration is the limiting step in the formation of the Sd-Vg complex and serves as the activating component, while Sd serves as the DNA binding component of the complex (Halder et al., 1998b; Simmonds et al., 1998). Based on *in vitro* data, it was suggested that Vg causes a conformational change in Sd so it interacts only with appropriate DNA target sequences (Halder and Carroll, 2001). The *in vivo* data presented in this thesis also agree with this notion.

Based on the *in vivo* data obtained, the following new observations can be made for the Sd-Vg complex with respect to its function in wing development. The N-terminal 87 amino acids of Sd are dispensable whereas the rest of the protein is necessary for normal wing development, since Δ1-87 was the only construct to behave as full length Sd in the wing rescue assay. The linker domain serves an important, yet unknown, function in this tissue. Expression of constructs that interrupted or replaced the linker domain of Sd caused these proteins to behave in a different manner than full length Sd in the wing rescue assay. Whatever function(s) the linker domain has requires the entire Sd protein except for the N-terminal 87 amino acids. Evidence to support this is that fusing the linker domain to only the TEA DNA binding domain, or the VID, or the C-terminal domain had no effect in the wing. What is clear is that the linker domain is not required for Vg to interact with Sd. If the linker domain is involved in Vg interaction, Sd with interruptions or replacements of this linker region would be predicted to have no effect in the wing rescue assay, and this is not what is observed. The VID and the entire C-terminal domain of Sd are required for a stable interaction between Sd and Vg. Any Sd proteins with truncations in the VID or C-terminal domain had no effect in wing development. This is presumably due to the failure of these mutant Sd proteins to titrate limiting Vg away from endogenous Sd. Besides the full length protein, the only Sd deletion construct that was able to cause ectopic expression of endogenous *sd* in the third instar wing disc was Δ1-87. This serves as additional evidence to suggest that amino acids 88 to 440 of Sd are required for its role in development of the wing. Furthermore, the ability of Sd to cause ectopic endogenous *sd* expression is also dependent on Sd concentration.

Sd may have one or several functions in the eye-antennal imaginal disc, and there may be a link between Sd and cell growth regulation in the eye. It is likely to interact with an unknown factor (X) for proper formation of the eye.

Constructs Δ1-87 and full length Sd were able to cause lethality in the fly when expressed via the *ptc*Gal4 driver. This indicates that the N-terminal 87 amino acids of Sd are not required for the lethal effect of ectopically expressed *sd*. However, it remains uncertain why *sd* constructs with interruption of the linker domain also lead to lethality of the fly. What is known is that as with the N- terminal 87 amino acids of Sd, the linker region is also not required for the lethal effect exerted by the full length protein.

There are parallels and differences between the effects of Sd in wing and eye development. With respect to the role of Sd in wing development, full length and Δ1-87 Sd are able to cause either a rescue or a dominant negative effect in the rescue assay. Presumably, this is due to a difference in expression levels between each independent transgenic line of the respective construct. In contrast, full length Sd and Δ1-87 had no effect in the eye over-expression assay. It is uncertain why this is the case. However, the different outcomes between the two assays may be attributed to the formation mechanism of Sd-Vg and putative Sd-X in the wing and eye tissues, respectively. One speculation is that Sd and X form in a concentration independent manner whereas formation of the Sd-Vg complex in the wing is dependent on the relative concentration of the two proteins. Interrupting the TEA domain and linker causes a phenotype in both the wing and eye, while interrupting the VID and C-terminal domain has no effect in the two tissues. These results are consistent with the notion that Sd interacts with its DNA targets via the TEA DNA binding domain and associates with its co-factor(s) via the VID and C-terminal domain in both the wing and eye. Furthermore, this demonstrates that the linker also has a function in wing and eye development.

In addition to the possible role of Sd in development of the wing and eye tissue, there is evidence to suggest that Sd also has a role in leg and heart development. With respect to the possible role of Sd in leg development, over-expression of Sd Δ1-200 can cause a duplication of the leg or kinks within the tibia of the leg (Garg et al., 2006) and sd^{ETX4} reporter activity is found in the jump muscle of the mesothoracic leg (Campbell et al., 1992). In regards to the possible role of Sd in heart development, over-expression of Sd was able to alter expression of *ladybird* (*ld*), a gene required to specify the identity of heart precursors in the cardiogenic *tinman*-cascade (Jagla et al., 1997; Bidet et al., 2003). It is possible that the function of Sd evolved to perform distinct functions in several *Drosophila* tissues and that Sd interacts with different co-activators to promote proper development of these tissues. This is made possible by separating the DNA binding domain and the activating domain of a transcription factor, such that while Sd provides the DNA binding component, another protein is required to provide the activating function. In the case of the wing, we know this co-activator to be Vg, while the identity of the partner protein in other tissues is as of yet unknown. The highly conserved TEA DNA binding domain is likely required for Sd to bind various targets while components of the VID and C-terminal region are required for it to interact with different co-factors. Perhaps in various tissues the binding of different factors can alter Sd target selectivity allowing it to perform various roles in development (Mann and Carroll, 2002).

6.2 The evolutionary advantages provided by Sd

The evolution of a general transcription factor such as Sd with its DNA binding component separated from its activating component can provide several advantages. First, this provides another level of control such that it is only when Sd is present with its specific co-factor in the same temporal and spatial pattern that the desired target genes are activated. Second, at least one component (Sd in this case) of the Sd-co-activator complex can be "reutilized" to carry out additional functions by means of interaction with different tissue specific co-factors (Srivastava et al., 2002). For example, Sd is likely to interact with an unknown co-factor in the eye for the development of this tissue.

6.3 The Sd-Vg complex requires other inputs to activate wing target genes

Selector input by the Sd-Vg complex requires other signals such as the N signaling pathway to promote wing fate in a precise temporal and spatial manner (Guss et al., 2001). This theory proposes that it is only when Sd and Su(H) (transcription factor for the N pathway) bind cis-regulatory elements on target genes that there will be activation of transcription (Guss et al., 2001). It has been suggested that this may be a common mechanism by which signaling pathways can elicit tissue or field specific responses (Affolter and Mann, 2001).

6.4 Unanswered questions

There are still many unanswered questions regarding how the Sd-Vg complex functions in wing development and the possible roles of Sd in development of other tissues. With respect to Sd in wing development, it is still uncertain if Sd-Vg forms a multi-protein transcriptional complex with other factors to promote proper wing development? Can Vg be involved in recruiting these other factors to the Sd-Vg complex? If additional potential partners of the Sd-Vg complex can be determined, this will provide insight into the mechanism by which this complex activates target gene expression in the wing disc. Furthermore, the Sd dimerization domain has not yet been localized. The answer to this question should help us to understand how the Sd-Vg complex is formed. The putative NLS of Sd has never been defined *in vivo*. It may be possible to characterize the region required to localize Sd into the nucleus by fusing the putative nuclear localization signal (NLS) to GFP and assaying for the ability of GFP to enter the nucleus.

Another very important question is what are the possible role(s) of Sd in the eye imaginal disc? Using various computer programs, attempts have been made to find *Drosophila* proteins that share sequence homology with regions of Vg that interact with Sd. Presumably, this can find proteins that may have the ability to interact with Sd. Unfortunately, searches so far have not identified any putative partners of Sd. Therefore, the next logical step would be to find possible partners of Sd in this tissue via methods such as GST-pull down experiments or the Yeast two hybrid assay. Another question that has not been addressed is whether the simultaneous over-expression of the tumor suppressor p53 protein or the anti-apoptotic protein p35 would have any effect in alleviating or enhancing the eye outgrowth phenotype. Answers to this question may provide stronger evidence to link the eye phenotype to defects in cell growth regulation.

Similarly, the over-expression assay done in the eye can be carried out in the leg to determine which region(s) of Sd can affect leg development. In turn, the partners of Sd in leg development may then be determined. With respect to the possible role of Sd in heart development, the staining pattern of *ld* can be compared between wildtype *Drosophila* larvae and those harboring lethal mutations of the *sd* gene. This could provide more insight into how Sd can be affecting *ld* expression, and thus the possible function of Sd in heart development.

Further dissection of the domains of Sd important for its vital function can also be examined. One way to address this question would be to use the collection of Sd constructs to rescue *sd* lethal alleles. A complementary experiment would be to over-express these constructs to determine which region(s) of Sd are still able to cause lethality in the fly.

Another question that has not been addressed is whether post-translational modifications such as phosphorylation are involved in regulation of Sd function. Interestingly, it has been shown that phosphorylation of TEF-1 can alter its DNA binding affinity (Gupta et al., 2000). The amino acid involved in this phosphorylation is serine residue #102 located immediately downstream of helix 3 of the TEA domain of TEF-1 (Gupta et al., 2000). By using a computer analysis program, several putative phosphorylation sites of Sd were found (Blom et al., 1999) (Figure 6.1). In total, there are five putative serine and two putative tyrosine phosphorylation sites. The serine residues are at amino acid positions: #106, #149 (TEA DNA binding domain), #207 (linker region), and #324, #328 (VID) of Sd. The two tyrosine residues are at amino acid positions #247 and #289 of Sd, where the VID is situated. Are any of these putative sites phosphorylated? *In vitro* experiments can be performed to determine if phosphorylation of Sd can alter its DNA binding affinity. For example, does increasing the concentration of a protein kinase such as protein kinase A (PKA-found to phosphorylate TEF-1) (Gupta et al., 2000) have an effect on the ability of Sd to bind target probes? *In vitro* protein-protein interaction assays could also be performed to determine if phosphorylated Sd has any altered ability to associate with Vg. If phosphorylation is found to affect one or both of these processes, this can lead to experiments that will be done *in vivo* to address the following questions. Can the putative phosphorylation sites in the TEA DNA binding and linker region of Sd be involved in its ability to bind DNA targets? Can phosphorylation of serine #324 and #328 in the VID be involved in Sd-Vg interaction? Coincidentally, these two residues are conserved between Sd and TEF-1 and are also predicted to be phosphorylated in TEF-1 (Figure 6.1). Furthermore, can phosphorylation

Figure 6.1: Diagram of the putative phosphorylation sites found in Sd. The sequences in red, green, and blue represent the TEA DNA binding domain, linker, and VID respectively. The black arrows indicate the putative phosphorylation sites of Sd while the red arrows represent the two putative serine residues that are predicted to be phosphorylated in both Sd and TEF-1.

```
MKNITSSSTC STGLLQLQNN LSCSELEVAE KTEQQAVGPG TIPSPWTPVN AGPPGALGSA
                                                 ↓
DTNGSMVDSK NLDVGDMSDD EKDLSSADAE GVWSPDIEQS FQEALSIYPP CGRRKIILSD
                              ↓
EGKMYGRNEL IARYIKLRTG KTRTRKQVSS HIQVLARRKL REIQAKIKVQ FWQPGLQPST
                            ↓
SQDFYDYSIK PFPQPPYPAG KTSTAVSGDE TGIPPSQLPW EGRAIATHKF RLLEFTAFME
      ↓                                             ↓
IQRDEIYHRH LFVQLGGKPS FSDPLLETVD IRQIFDKFPE KSGGLKDLYE KGPQNAFYLV
                         ↓   ↓
KCWADLNTDL TTGSETGDFY GVTSQYESNE NVVLVCSTIV CSFGKQVVEK VESEYSRLEN

NRYVYRIQRS PMCEYMINFI QKLKNLPERY MMNSVLENFT ILQVMRARET QETLLCIAYV

FEVAAQNSGT THEIYRLIKE
```

be another mechanism to regulate Sd function in different tissues? To test some of these theories *in vivo*, targeted mutagenesis can be used to create mutations in these putative phosphorylation sites and determine if they have any effect in development of the wing, eye, or leg. For example, the putative phosphorylation sites in the TEA DNA binding domain or the linker region can be replaced with neutral amino acids such as glycine and injected into *Drosophila* embryos to make transgenic flies. Presumably, this will eliminate phosphorylation at these targets. What effects will these constructs have in the wing rescue assay, or the eye over-expression assay? The same experiment can be performed with the putative sites in the VID to determine if mutating the putative phosphorylation sites in that region can alter the ability of Sd to form a stable complex with Vg.

In conclusion, the work presented suggests that Sd has diverse roles in *Drosophila* development, and the role of Sd may not be limited to the tissues discussed above. Vg has been shown to specify the differentiation of the *Drosophila* indirect flight muscles (Sudarsan et al., 2001). A Gal4 enhancer trap allele of *sd*, $sd^{29.1}$ shows reporter activity in the indirect flight muscles of the adult thorax (Shyamala and Chopra 1999). Could Sd be also involved in development of the indirect flight muscles? In addition to its role in heart cell specification, *ld* is also required for the identity of the segmental border muscle and the lateral adult muscle precursors (Jagla et al., 1998). What is the relationship between Sd and Ld? Does Sd also have a role in promoting muscle cell fates? Evidently, there are still many more unanswered questions about the diverse roles of Sd in *Drosophila* development.

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